

HYPOTHYROID-INDUCED DIAPHRAGMATIC DYSFUNCTION:
BIOCHEMICAL AND CONTRACTILE ALTERATIONS

BY

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DEDICATION

To Denise, for your love, support, and understanding over the past four years.
Your presence has been a constant source of strength and encouragement.

&

To my parents, for instilling in me faith, hope, and love. Thank you for
giving me the freedom and means to achieve my dreams.

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Abstract of Dissertation Presented to the Graduate School
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HYPOTHYROID-INDUCED DIAPHRAGMATIC DYSFUNCTION:
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By

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Major Department: Exercise and Sport Sciences

Recent data from our laboratory demonstrate that hypothyroidism in rats reduces *in vitro* peak diaphragmatic specific tetanic force (specific P_o) and maximal shortening velocity (V_{max}). At present, the mechanism(s) responsible for these alterations are unknown. Therefore the purpose of this study was to improve our understanding of the cellular mechanism(s) responsible for hypothyroid-induced diaphragmatic contractile dysfunction. Specifically, this project tested the following hypotheses: 1) the reduced diaphragmatic specific P_o in hypothyroid animals is due to alterations in the intrinsic contractile properties of the myofibril and 2) the reduction in diaphragmatic V_{max} parallels a decrease in myosin ATPase activity and a fast to slow shift in myosin heavy chain (MHC) distribution.

To test these hypotheses, *in vitro* isometric and isotonic contractile properties were measured in costal diaphragm strips of adult, female Sprague-Dawley rats. In addition, diaphragmatic skinned single fibers (types I and II) were studied for maximal calcium-activated specific force (specific F_0) development and calcium sensitivity. Animals were divided into two groups: hypothyroid ($n=13$) and control ($n=12$). Hypothyroidism was induced by surgical thyroidectomy and daily injections of n-propylthiouracil over a six week period.

Results indicated that diaphragmatic specific P_0 was reduced 21.1% in the hypothyroid animals ($p<0.05$), while specific F_0 of both type I and II costal diaphragm fibers did not differ between groups ($p>0.05$). Further, diaphragmatic V_{\max} decreased 25% in hypothyroid diaphragms compared to controls ($p<0.05$). This reduction in V_{\max} was accompanied by a concomitant decrease in diaphragmatic ATPase activity ($p<0.05$) and a fast (type IIb) to slow (type I) MHC isoform shift.

These data do not support the hypothesis that the hypothyroid-induced decrease in diaphragmatic specific P_0 can be explained by intrinsic changes in the maximal force generating capacity of the individual myofibril. Rather, these findings suggest that alterations in some step(s) of excitation-contraction coupling is/are responsible for the diaphragmatic specific force deficit. Finally, a strong correlational relationship exists between *in vitro* diaphragmatic V_{\max} and myofibrillar ATPase activity ($r=0.80$; $p<0.05$), thus supporting the second hypothesis.

CHAPTER 1 INTRODUCTION

Hypothyroidism exerts a profound influence on the mechanical and biochemical properties of striated muscle. Of particular importance is that hypothyroidism results in impaired respiratory muscle function and dyspnea in humans [1-4]. Recent data from our laboratory suggest that hypothyroidism results in contractile dysfunction of the rat diaphragm (unpublished data). Specifically, we have demonstrated *in vitro* that maximal isometric tetanic specific tension (specific P_o) of the costal diaphragm is reduced ~20% ($p < 0.05$) and maximal shortening velocity (V_{max}) slowed ~30% ($p < 0.05$) in response to the hypothyroid state. The mechanism(s) by which hypothyroidism induces such mechanical alterations in the diaphragm is currently unknown and will be the focus of the proposed experiments.

Several rival hypotheses could explain the observed impairment in hypothyroid-induced diaphragmatic specific P_o , namely, 1) a reduction in myofibrillar protein concentration (cross-bridge content per muscle cross section), 2) maximal force generation per crossbridge (i.e. reduction in maximal calcium (Ca^{2+}) activated tension), 3) alterations in excitation-contraction (E-C) coupling or 4) some combination thereof. Previously, we have demonstrated that the deficit in diaphragmatic specific P_o is not due to a reduction in myofibrillar protein concentration (unpublished data). This finding is in agreement with studies measuring normalized myofibrillar content in hypothyroid limb muscle [5, 6].

The primary purpose of this study was to test the hypothesis that hypothyroidism reduces diaphragmatic specific P_o by altering the intrinsic contractile properties of the isolated single fiber. To test this hypothesis, chemically-skinned myofibrils from the hypothyroid costal diaphragm were studied to assess maximal Ca^{2+} activated force (F_o) and Ca^{2+} sensitivity. Further, the relationship between hypothyroid-induced shifts in myosin isoforms (i.e. alterations in myosin ATPase activity) and changes in V_{max} was examined. The rationale for this experimental approach is discussed in the forthcoming hypothesis justification section.

Significance

While it is well established that hypothyroidism results in alterations in locomotor muscle phenotype and contractile function, studies involving respiratory muscles are few. At present, little information is available concerning the cellular impact of thyroid-deficiency on the mammalian diaphragm. Specifically, to date, no data exist describing the cellular alterations associated with diaphragmatic contractile dysfunction following hypothyroidism; therefore, the mechanism(s) responsible for alterations in contractile function in response to thyroid-deficiency are unknown. Thus, improving our understanding of the relationship between thyroid status and the cellular mechanism(s) responsible for alterations in contractile function will expand our knowledge on the role of thyroid hormone in the regulation of contractile and biochemical properties of the mammalian diaphragm.

Specific Aims

Experimental hypothyroidism has been shown to alter *in vitro* contractile properties of the costal diaphragm (unpublished data). The reported study was designed to determine the mechanism(s) responsible for hypothyroid-induced diaphragmatic contractile dysfunction based on an understanding of its cellular pathophysiology utilizing a rodent model. Costal diaphragms of hypothyroid animals were compared to diaphragms from controls to answer the following questions.

Primary Question

Is the hypothyroid-induced specific force (P_o) deficit in the costal diaphragm strip, due to some alteration in the intrinsic contractile properties (maximal Ca^{2+} -activated force) of the isolated myofibril?

Primary Hypothesis

The reduced P_o of the hypothyroid costal diaphragm is principally due to alterations in the intrinsic nature of the myofibril (decreased maximal Ca^{2+} -activated force).

Secondary Question

Does the hypothyroid-induced reduction in diaphragmatic maximal shortening velocity (V_{max}) parallel decreases in myosin ATPase activity (fast, type IIb MHC to slow, type I MHC)?

Secondary Hypothesis

The reduced V_{max} of the hypothyroid costal diaphragm will be highly correlated to a decrease in myosin ATPase activity (fast, type IIb MHC to slow, type I MHC shift).

Hypothesis Justification

Isometric Specific Force Production

We have previously demonstrated a reduction in specific P_o of hypothyroid costal diaphragm muscle strips. At present, however, the mechanism(s) to explain this contractile deficit are unknown. As mentioned previously, three potential mechanisms or some combination thereof may explain the force deficit observed in the *in vitro* hypothyroid costal diaphragm. Specifically, a preferential decrease in myofibrillar protein content per muscle cross section, alterations in the intrinsic contractile properties of the myofibril (e.g. maximal Ca^{2+} -activated force), and altered excitation-contraction (E-C) coupling could independently or in combination result in diminished specific force production. Since we have demonstrated that a reduction in myofibrillar protein concentration is not responsible for the hypothyroid-induced force deficit (unpublished data), this study will focus on the alternative hypothesis that hypothyroidism results in intrinsic changes in the force generating capacity of the isolated myofibril.

To determine if alterations in the contractile function of isolated hypothyroid myofibrils explains the force deficit seen in the *in vitro* muscle strip, the intrinsic properties of the myofibril must be separated from factors related to E-C coupling. This was achieved by chemically permeabilizing the sarcolemma and sarcoplasmic reticulum (SR) from isolated single muscle fibers. This approach allows the direct activation of the contractile apparatus, hence by-passing E-C coupling. In these measurements, the contractile process was experimentally manipulated by controlling the free Ca^{2+} concentration in the solution surrounding the myofibril. Thus it can be indirectly determined

whether the hypothyroid-induced force deficit is due to some intrinsic property of the contractile apparatus or involves some phase of E-C coupling.

The rationale for determining maximal Ca^{2+} -activated force of hypothyroid fibers is as follows. Given that a reduced myofibrillar protein content does not contribute to the observed force deficit in the hypothyroid costal diaphragm, the possibility exists that hypothyroidism may induce intrinsic molecular changes within the muscle fiber that will lower force production per cross-bridge thereby reducing maximal Ca^{2+} -activated specific force (F_0). Therefore it was postulated that hypothyroidism would alter the intrinsic nature of the costal diaphragm contractile apparatus thereby decreasing specific force production. The finding of a hypothyroid-induced reduction in F_0 from single fibers will be interpreted as evidence that the observed decrease in specific P_0 is related to some intrinsic modification of the myofibril. In contrast, the observation that hypothyroidism does not alter specific F_0 would indicate that the decrease in maximal diaphragmatic specific force in hypothyroid animals is due to some factor other than intrinsic changes within the contractile apparatus (e.g. E-C coupling).

Maximal Shortening Velocity

In muscle, the rate of shortening is greatest when the fibers are completely unloaded (V_{\max}). It is generally agreed that the maximum rate of crossbridge cycling after the performance of a power stroke determines the speed of shortening [7]. Therefore, if the rate of ATP hydrolysis is reduced due to decreased myosin ATPase activity (fast to slow MHC shift), the rate of crossbridge attachment, and thus, muscle shortening is slowed. Support for this notion is based on the original work of Barany [8] who demonstrated a

strong positive correlation between V_{\max} and actomyosin ATPase activity of skeletal muscle homogenates. Subsequent studies utilizing single fibers from the rat soleus have revealed similar findings for both ATPase activity [9] and myosin heavy chain (MHC) distribution [10]. While decreases in V_{\max} have been reported for hypothyroid locomotor muscle [5, 11, 12], no studies have examined the influence of hypothyroidism on costal diaphragm V_{\max} . Further, information on the impact of hypothyroidism on diaphragmatic MHC profile and myosin ATPase activity is lacking. Therefore, these experiments tested the hypothesis that the hypothyroid-induced reduction in V_{\max} is highly correlated with changes in myosin ATPase activity and myosin heavy chain distribution.

CHAPTER 2 REVIEW OF RELATED LITERATURE

Hypothyroidism

The thyroid hormones (3,5,3'-triiodo-L-thyronine, T_3 ; thyroxine, T_4) are essential for normal cellular development, differentiation, and growth as well as the maintenance of numerous physiological processes [13]. Thyroid dysfunction manifests itself by an over- or underproduction of thyroid hormone. In hypothyroidism, the thyroid gland fails to maintain a level of thyroid hormone sufficient for normal body functions. In the majority of cases, the clinical syndrome results from cellular responses to a deficiency in circulating hormone levels. Such hormonal deficiency is frequently due to an abnormality of the thyroid gland (primary hypothyroidism). This is commonly the result of an autoimmune response directed against the thyroid gland. Patients initially develop thyroiditis followed by progressive glandular deterioration resulting in a diminished or absent secretion of thyroid hormone. Although much less common, thyroid-stimulating hormone (TSH) deficiency (secondary hypothyroidism) and thyrotropin-releasing hormone (TRH) deficiency (tertiary hypothyroidism) are also potential etiologies for hypothyroidism [14].

Clinical Incidence

Although hypothyroidism occurs in individuals of all ages, it is most common in individuals over 55 years old. The prevalence of hypothyroidism has been investigated in several cross sectional community studies and a number of more select population groups. Work by Tunbridge et al. [15] places the incidence in the United Kingdom between 7-8% of the population above 55 years of age while in the United States, the prevalence is estimated to be 4-7% of individuals over the age of 60 [16-18]. Worldwide, the incidence of skeletal muscle myopathy and weakness among patients with hypothyroidism has been estimated to vary between 30-80% [19]. Considering the relative incidence of primary hypothyroidism, particularly in the elderly, little information is currently available to elucidate the mechanisms responsible for the observed respiratory muscle dysfunction.

Clinical Manifestations

The clinical features of hypothyroidism are dependent upon the patient's age at onset as well as the duration and severity of the thyroid hormone deficiency. In its mildest form, hypothyroidism may be characterized by specific biochemical alterations, but clinical symptoms will be mild or absent. Alternatively, overt hypothyroidism of prolonged duration involves systemic pathologies including the respiratory, cardiovascular, and neuromuscular systems [13, 20]. Characteristically, however, clinical symptoms of thyroid deficiency develops insidiously over a prolonged period of time, due in part to the gradual yet progressive development of thyroid gland failure.

Some of the earliest symptoms associated with hypothyroidism involve the neuromusculoskeletal system [18]. Many individuals report locomotor muscle weakness and lethargy as common, but nonspecific complaints. Indeed, it is well established that hypothyroidism results in both respiratory and locomotor muscle dysfunction [1-6, 21]. As the clinical severity of hypothyroidism increases, an increased involvement of various organ systems including the respiratory system results. Common symptoms include dyspnea [2] and reduced inspiratory effort [1, 3]. Currently, limited information exists on the mechanism(s) responsible for the observed pathologies associated with the respiratory muscle system and its relationship to ventilatory function.

While it is universally agreed that hypothyroidism results in alterations in muscle function, evidence suggests that motor nerve neuropathy may also contribute to hypothyroid-induced muscle dysfunction. Although several investigators have suggested that hypothyroidism produces phrenic nerve neuropathy [1, 22], others have provided evidence that hypothyroidism does not alter neural conduction velocity, neuro-transmitter release, and/or muscle resting membrane potential [1, 23]. Such a lack of agreement among investigators may stem from differences in the degree of hypothyroidism between subjects and the time course of thyroid dysfunction prior to study. In both of the aforementioned investigations supporting the existence of hypothyroid-induced phrenic nerve neuropathy [1, 23], the authors concluded that the observed respiratory muscle weakness in hypothyroid patients was not due to neuropathy alone and that alterations in respiratory muscle function must also contribute significantly to diaphragmatic contractile dysfunction.

Hypothyroidism and Skeletal Muscle

Thyroid hormones exert profound effects on the growth, development, and homeostasis of mammalian muscle [24]. Although many structural and biochemical alterations have been identified in hypothyroid skeletal muscle of both humans and animals, the contribution of these factors to the impairment of muscle function remains unclear.

Impact on Adult Human Skeletal Muscle

Changes in the physiology and biochemistry of skeletal muscle are well documented under the clinical hypothyroid condition [25]. In man, thyroid dysfunction is commonly associated with muscle atrophy [4] and reduced inspiratory effort [1-3]. Further, muscle weakness and fatigue are commonly reported in hypothyroid patients [1, 2, 4, 25]. As mentioned earlier, clinical evidence of myopathy occurs in 30-80% of thyroid-deficient patients [26, 27]. Predominant features include muscle weakness, cramps, aching or painful muscles, and sluggish movements [28]. Although thyroid hormone replacement leads to complete clinical recovery in most cases, some individuals exhibit incomplete or delayed improvement [14, 28].

Contractile alterations

It is universally accepted that the hypothyroid state in both locomotor and respiratory muscle [4, 21, 29] results in a reduced *in vivo* muscle force generation in humans. [1,2, 4]. Specifically, Takamori et al. [30] demonstrated in hypothyroid patients a prolonged rate of tension development and reduced twitch force during *in vivo* contraction of the adductor pollicis muscle.

Similar contractile alterations (i.e. slowed contractile function and reduced force generation) in response to hypothyroidism are well characterized in rat locomotor muscle *in vitro*. [5, 12, 31-33]. The cellular mechanism(s) to explain these observations remain unclear and are the focus of the proposed experiments.

Biochemical alterations

In humans, complex and diverse physiological changes occur in response to the hypothyroid condition as evidenced histochemically by selective atrophy and loss of type II fibers [28, 34], enlargement of type I fibers [28], and glycogen accumulation [25]. These observations were confirmed by Wiles et al. [34] who showed that in hyperthyroid patients, the reverse effect was present.

Metabolically, hypothyroidism results in decreased activities of mitochondrial enzymes in human [35, 36] and rodent [37-39] skeletal muscle. This observation is more pronounced in slow-twitch (ST) red muscles than fast-twitch (FT) white muscle [38]. In addition, reductions in enzymes involved in glycogenolysis and glycolysis of hypothyroid animals [40, 41] have been reported. Such findings are consistent with the reduced lactate production found during ischemic exercise of the hypothyroid human forearm [42]. Further, more than 90% of patients with hypothyroidism exhibit elevated serum creatine phosphokinase levels suggestive of muscular involvement [43].

Finally, experimental evidence from hypothyroid muscle of man [25] suggests that adenosine triphosphate (ATP) production may be altered. This notion is supported by the *in vivo* study of hypothyroid patients by phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR). Argov et al.

{21} has suggested a hormone-dependent impairment in mitochondrial ATP synthesis both at rest and during exercise. Alternatively, Taylor et al. {44} argues that the inhibition of glycogenolysis during exercise is the principal bioenergetic abnormality, while alterations in intramuscular pH of resting and exercising hypothyroid muscle suggest abnormal proton handling within the mitochondria.

Impact on Adult Rodent Skeletal Muscle

The occurrence of physiological and morphological changes of rodent skeletal muscle in response to hypothyroidism is well documented {31-33, 38, 41, 45}. Studies on the influence of thyroid hormone deficiency on rat limb muscle have focused primarily on biochemical {21, 29, 46, 47} and structural {6, 39} alterations, or mechanical dysfunction {5, 12, 33}.

In the rat, alterations in thyroid status exerts profound influence on skeletal muscle characteristics, specifically fiber type transformation and alterations in energy metabolism. Research indicates that the hypothyroid state results in an increased proportion of ST (type I) fibers in mammalian locomotor muscle {48, 49}. Further, data suggest these changes to be more pronounced in postural muscles (e.g. soleus) than in FT (type II) muscles (e.g. extensor digitorum longus) {38, 39}. Such hypothyroid-induced alterations involve both reductions in myofibrillar ATPase activity and mitochondrial oxidative capacity {45, 49}.

Contractile alterations

The loss of normal thyroid function causes alterations in muscle components essential to the contractile process. In hypothyroid rats, changes

in the histochemical and biochemical profile of the soleus muscle have been reported by Ianuzzo et al. [50] and Nwoye et al. [49]. Further, experiments using the hypothyroid soleus have shown prolongation of both time to peak tension (TPT) and one-half relaxation time ($RT_{1/2}$) during isometric contractions [5, 33]. Such contractile changes of the hypothyroid soleus are correlated to shifts in the histochemically-defined fiber type profile [51]. Isotonic properties of the hypothyroid soleus, namely a decrease in V_{\max} and reduced maximal rate of tension development, have also been reported [5, 11, 12]. Despite the use of various biochemical and physiological techniques in locomotor muscle, relatively little is known about the direct effects of hypothyroidism on the *in vitro* costal diaphragm strip or isolated single fiber.

Biochemical alterations

Hypothyroidism is associated with marked alterations in skeletal muscle mitochondrial structure and function. For example, mitochondria isolated from hypothyroid animals exhibit decreased respiration rates when compared to euthyroid controls [52]. Further, previous reports have demonstrated that thyroid deficiency results in marked reductions in skeletal muscle enzyme activities [29, 38, 39, 53] as well as mitochondrial components, including cristae elements (cytochrome c) [47] and the capacity of isolated mitochondria to oxidize various substrates [46, 54, 55]. Such observations seem contradictory since mitochondrial yields are similar between control and thyroid-deficient muscle [53]. Thus, it appears that alterations in mitochondrial composition, rather than a reduction in mitochondrial volume, may be responsible for the reduced respiratory capacity of hypothyroid skeletal muscle. This argument seems plausible given that thyroid hormone is thought to control proton conductance across the mitochondrial inner membrane by modifying

membrane surface area and/or fatty acid composition [52]. These modifications could potentially explain the reduced proton leak across the inner mitochondrial membrane seen in hypothyroid animals [52].

Myosin isoform expression

Myosin is the major constituent protein of the skeletal muscle contractile apparatus. Indeed, myosin plays a central role in the transduction of chemical to mechanical energy within mammalian muscle systems. Native myosin (~500 kDa) exists as a hexameric, asymmetric protein arranged into two globular heads attached to a long alpha-helical rod-like portion. The rod portion is responsible for the assembly of myosin into thick filaments while the two globular heads contain both the enzymatic active site and actin-binding region.

The subunit structure of myosin is composed of two identical heavy chains (MHC) (~200 kDa) and two pairs of distinct light chains (MLC) (~14-20 kDa). The light chains consist of a pair of phosphorylatable (regulatory) light chains (LC2) and a pair of alkali (essential or catalytic) light chains (LC1 and LC3). Although the physiological role of the light chains is unclear, their position near the hinge region suggests that they may be involved in modulating myosin/actin interactions [56]. Like other ubiquitous proteins, myosin is regulated by a highly conserved multigene family [57]. Although functionally diverse, the molecular structure and subunit composition of most myosins is quite similar. Alternatively, it appears that the primary structure of the various isoforms gives rise to its physiological diversity.

In the adult mammalian costal diaphragm, four myosin heavy chain (MHC) isoforms exist. Relative to their migration pattern (slowest to fastest) when separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-

PAGE) they are: 1) type IIa; 2) type II_{d/x}; 3) type II_b; and 4) type I. Specifically, the MHC composition in the costal diaphragm of female Sprague-Dawley rats consists of: type IIa MHC = ~20%; type II_{d/x} MHC = ~40%; type II_b MHC = ~15%; and type I MHC = ~25% (unpublished observation). The MHC composition of the diaphragm is significant since it is well established that the MHC isoform contained within individual muscle fibers determines specific contractile properties of the muscle [58]. For example, Eddinger and Moss [59] have demonstrated that single diaphragmatic muscle fibers containing type II_b or II_{d/x} MHC's have significantly greater maximal shortening velocities compared to fibers containing type I or IIa MHC isoforms.

The thyroidal influence on skeletal muscle myosin is well known. Indeed, numerous experiments have demonstrated that an animal's thyroid status modulates both mRNA and protein expression of MHC as well as MLC composition and myosin ATPase activity [6, 48-50, 60]. The thyroid hormones act through nuclear receptor proteins that repress or activate the transcription of myosin genes [61]. Recent studies have demonstrated that the decrease in plasma thyroid hormone concentration associated with hypothyroidism alters locomotor muscle MHC phenotype resulting in a shift from type II_b to type I MHC (fast to slow isoform transition) [5, 6]. Indeed, hypothyroidism is clearly associated with the down-regulation of adult fast (type II) myosin isoform synthesis and the expression of the slow (type I) isomyosin in skeletal muscle [45, 48, 50, 62]. Moreover, Izumo et al. [63] has demonstrated that hypothyroidism modulates the expression of MHC mRNA in a tissue-specific manner. Specifically, in the ST soleus muscle (~90% type I MHC), hypothyroidism results in an increased expression of the MHCI mRNA while completely inhibiting the expression of MHCIIa mRNA. Alternatively,

hypothyroidism induced in FT muscle produces only slight increases in type MHCIIa mRNA expression.

Thyroid hormone-mediated shifts in skeletal muscle MLC composition and myosin ATPase activity have also been reported by several groups. Johnson et al. [64] reported a decrease in fast MLC expression of the hypothyroid soleus muscle, while Nwoye et al. [49] demonstrated reductions in both Ca^{2+} and Mg^{2+} -activated ATPase activity. This finding is important since Hoh [65] has shown that the underlying basis of myosin ATPase activity changes is the induction or suppression of myosin isoform expression.

Thus, while it is well established that slow muscle (e.g. soleus) is more responsive to reduced levels of circulating thyroid hormone than fast muscle (due to an increased thyroid receptor density) [66], the impact of hypothyroidism on the MHC profile of the costal diaphragm (mixed fiber type) is currently unknown.

Hypothyroidism-Induced Dysfunction in Human Respiratory Muscle

Skeletal muscle weakness is a recognized complication of hypothyroidism. Respiratory manifestations of hypothyroidism include dyspnea on exertion and exercise intolerance [67, 68], decreases in ventilatory responses to hypoxia and hypercapnia [69-71], pleural effusions [69, 72], and decreased maximal inspiratory pressures [73].

Although respiratory muscle weakness in hypothyroid patients is a common clinical observation [1-4, 22, 74], quantitative analysis of hypothyroid-induced diaphragmatic dysfunction have only recently been reported. Specifically, several reports have demonstrated that hypothyroid patients exhibit reduced diaphragmatic strength and endurance. Maximal

transdiaphragmatic pressure in hypothyroid patients ranged from 2 to 64 cm H₂O while normal values range from 100 to 160 H₂O {1, 2}.

Hypothyroidism and the Adult Rodent Diaphragm

The diaphragm is the primary inspiratory muscle in mammals and is the only skeletal muscle considered essential for normal ventilation {75}. The diaphragm can be divided into two discrete portions, the crural and costal, which are functionally and metabolically distinct {75, 76}. In mammals, the two regions exhibit different mechanical actions on the chest wall, while in the rat, the costal portion exhibits a greater oxidative capacity (+20%) when compared to the crural {75, 77}. Such an observation is likely due to the preferential recruitment of the costal portion during normal ventilatory activities in the rat {75}. Correspondingly, the costal region comprises ~70% of the total diaphragmatic mass {75}. Therefore, due to the significant contribution of the costal region in normal ventilation, the proposed experiments will focus on this region. Hereafter, reference to the diaphragm will be specific to the costal portion.

Of the studies investigating the influence of experimental hypothyroidism on skeletal muscle, only four have examined the adult mammalian diaphragm. Izumo et al. {63} examined the relationship between thyroid status and MHC gene regulation in a variety of muscles including the diaphragm. In hypothyroid animals, diaphragmatic type IIb MHC gene was down-regulated, while the expression of the type I MHC gene remained unchanged. Interestingly, hypothyroidism also induced the re-expression of the embryonic MHC mRNA in the adult rodent diaphragm.

Ianuzzo et al. [29] demonstrated major alterations in the energy transducing capacity of the hypothyroid diaphragm, namely significant reductions in the enzymatic potentials for glycolysis (lactate dehydrogenase, phosphofructokinase), Krebs cycle (succinate dehydrogenase), and fatty acid oxidation (3-HADH). Histochemically, a shift towards slow fiber phenotype (type I) was reported, which is consistent with data from rodent locomotor [39] and respiratory (costal diaphragm) [62] muscles.

Finally, Denys and Hoffman [31] evaluated the influence of hypothyroidism on diaphragmatic twitch and tetanic contractile characteristics. They determined that the costal diaphragm responds to hypothyroidism in a manner similar to that of locomotor muscle (i.e. general slowing of contractile function). Further, they concluded that the *in vitro* diaphragm preparation was an appropriate experimental technique providing results similar to the *in vivo* clinical condition.

These findings demonstrate that hypothyroidism modifies the mechanical and phenotypic profile of the adult costal diaphragm, specifically regulation of MHC gene expression, fiber type distribution, and enzymatic capacity. Clearly, such cellular modifications may potentially alter diaphragmatic contractile function.

CHAPTER 3 METHODS AND PROCEDURES

Animals and Experimental Design

This study was approved by the University of Florida Institutional Animal Care and Use Committee (#2386) and followed the guidelines for animal care established by the American Physiological Society.

Experiments were performed on adult (120 day old) female Sprague-Dawley (SD) rats ($n=25$) weighing 250-300 grams. Animals were housed two per cage and maintained on a 12 hour photoperiod with standard rat chow and water available *ad libitum*. To standardize environmental influences and human contact, animals were handled daily by trained laboratory personnel 14 days prior the beginning of experimentation.

Hypothyroidism was induced by surgical thyroidectomy and daily intraperitoneal (IP) injections of the antithyroid drug 6-n-propyl-2-thiouracil (PTU) over a six week period. Control animals received isotonic saline (pH=10.5) over the same six week time period. To ensure maintenance of Ca^{2+} homeostasis [78], thyroidectomized animals were administered 2% calcium lactate in their drinking water *ad libitum* over the initial ten day period following surgery. Rats were matched for initial body weights and assigned to one of two groups based on thyroid status:

Group 1: Control, euthyroid (C) (saline) ($n=12$)

Group 2: Hypothyroid (H) (thyroidectomy + PTU; $12.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) ($n=13$)

The decision to assign 12-13 animals per group was based on a statistical power analysis using data from pilot experiments (unpublished observation). The decision to use a six week experimental treatment period was based on the work of others [5, 6, 48] as well as preliminary experiments from our laboratory (unpublished observation) which have demonstrated that six weeks is sufficient to modulate muscle fiber phenotype and contractile function in both locomotor and respiratory muscle.

Choice of Animal Model

The rat was chosen as the experimental model because 1) an extensive body of literature involving the use of rats in thyroid hormone research is available; 2) many of the hypothyroid-induced changes in skeletal muscle are similar between human and rat; 3) the rat appears to be an excellent model to study respiratory muscle function since the rodent diaphragm is similar to the human diaphragm in terms of function and fiber type composition [79]; and 4) the acute and invasive nature of the present experiments prevents the use of human subjects. The female Sprague-Dawley rat was specifically chosen because 1) previous pilot work has demonstrated Sprague-Dawley rats to be an acceptable animal model for respiratory muscle studies and 2) this stock and strain have been characterized under well-defined conditions with respect to locomotor and respiratory muscle properties. [75, 80-83].

Experimental Objectives

The primary objective of this study was to test the hypothesis that the previously demonstrated force deficit observed in the *in vitro* hypothyroid costal diaphragm strip is due to an intrinsic alteration in the contractile function of the isolated hypothyroid costal diaphragm single fiber. This postulate was tested by comparing the force deficits between *in vitro* diaphragm strips and isolated skinned fibers. Further, the relationship between hypothyroid-induced shifts in myosin isoforms (i.e. alterations in myosin ATPase activity) and changes in V_{\max} was assessed. To achieve these objectives the following procedures and measurements were performed.

Induction of Hypothyroid State

Thyroidectomy and Chronic n-Propylthiouracil Treatment

A complete surgical thyroidectomy was performed by the vendor (Harlan Sprague-Dawley) on each animal prior to shipment to the University of Florida Animal Resource Center. Additionally, PTU was administered to thyroidectomized animals to ensure complete ablation of any residual thyroid tissue following surgery. This approach has been used effectively by others [5] as well as in preliminary experiments from our laboratory.

PTU is a synthetic reversible anti-thyroid goitrogen which inhibits thyroidal biosynthesis of both T_3 and T_4 [84]. Specifically, PTU prevents the formation of thyroid hormone from precursor iodides and tyrosine. The drug exerts this effect by inhibiting both tyrosine iodination and iodotyrosine coupling within the thyroid gland [85].

PTU was solubilized in 0.9% normal saline (pH=10.5) vehicle at a concentration corresponding to 6.0 mg/ml. The prepared PTU solution was administered daily via intraperitoneal injection to the hypothyroid group while control animals received normal saline only. All injections (~0.6 ml) were administered at approximately the same time of day in each group.

Assessment of Hypothyroid State

Thyroid status was verified by several established parameters previously defined in the literature [5, 6, 48]. They included:

Plasma triiodothyronine determination

Blood samples were obtained via direct cardiac puncture for assessment of circulating free T_3 levels at the termination of the experimental period (day 42). Samples were centrifuged to remove formed elements and plasma aliquots stored at -80°C until analysis by standard radioimmunoassay (RIA; Diagnostic Products, Los Angeles, CA). Samples were analyzed in duplicate and assayed on the same day to avoid inter-assay variability.

Measurement of resting metabolic rate

Measurements of resting oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were made during the fifth week of the experimental period to document the effectiveness of the thyroidectomy and PTU treatment on reducing metabolic rate. Animals were placed in a Plexiglass flow-through sampling system with a flow rate of 5.0 l/min. Animals were allowed a two hour acclimitization period in the sampling chamber prior to metabolic determination [86]. Expired oxygen and carbon dioxide fractions

were analyzed using Sensor Medics sensors (Anaheim, CA) and calculated using standard procedures. Analyzers (oxygen and carbon dioxide) were calibrated prior to each measurement with known gas standards.

Cardiac mass and native myosin isoform profile

After reaching a surgical plane of anesthesia, hearts were rapidly excised, trimmed free of the great vessels and weighed. Left ventricular samples were homogenized in sodium pyrophosphate (pH=8.8), glycerinated and stored at -20°C until analysis. Native isomyosin profile was determined by non-dissociating polyacrylamide gel electrophoresis. It is well documented that hypothyroidism promotes a reduction in heart mass and a V₁ to V₃ cardiac isoform shift in the rat [87]. For example, Fitzsimons et al. [48] noted a 20-fold increase in the low myofibrillar ATPase V₃ isoform of left ventricles from hypothyroid rats.

In vitro Contractile Properties in Costal Diaphragm Strips

Tissue Removal

Twenty-four hours after the final PTU injection, animals were weighed and anesthetized with 90.0 mg•kg⁻¹ pentobarbital sodium intraperitoneally. After a surgical plane of anesthesia was reached, the diaphragm and attached chest wall was removed *in toto* and quickly placed in oxygenated Krebs-Henseleit solution in preparation for *in vitro* contractile measurements. A strip of the ventral portion of the costal diaphragm (~3.0 mm x 20-30 mm) was initially removed for the assessment of *in vitro* contractile function (see section below). The remaining portions of the diaphragm were trimmed free

of fat and connective tissue (excluding central tendon), blotted dry, and weighed. Subsequently, the costal diaphragm was divided into four sections: 1) frozen in liquid nitrogen and stored at -80°C for measurement of myofibrillar protein concentration, Ca^{2+} -activated myofibrillar ATPase activity, and MHC isoform distribution; 2) weighed and frozen in liquid nitrogen for analysis of muscle water content; 3) frozen in liquid nitrogen for determination of connective tissue concentration; and 4) stored in relaxing solution at 5°C for single fiber contractile measurements.

In vitro Tissue Preparation and Experimental Set-up

The excised *in toto* diaphragm was immersed in oxygenated Krebs-Henseleit solution within 1 minute. Muscle strips were dissected from the ventral portion of the costal diaphragm. Briefly, the origin of the muscle with the rib intact was mounted by tissue clip onto a movable plexiglass support system and placed into a tissue bath (Harvard Scientific) containing curarized Krebs solution. The central tendinous region was clipped and attached to the lever arm of a dual-mode servo ergometer (Series 300B; Cambridge Technologies). The Krebs solution in the tissue bath was bubbled with a 95% O_2 -5% CO_2 gas mixture and maintained at a pH of 7.48 ± 0.05 and osmolality of ~ 290 mOsmol. Organ bath temperature was maintained at $24.5 \pm 0.5^{\circ}\text{C}$ since higher temperatures result in deterioration of muscle function [88]. $12\mu\text{M}$ d-tubocurarine was added to the Krebs solution to ensure complete neuromuscular junction blockade.

The mounted muscle strip was manipulated by the use of a micrometer-controlled platform. Muscle fiber length was adjusted by moving the position of the support in relation to the ergometer which was used to measure and

control muscle load and displacement during both isometric and isotonic contractions. Data output was obtained and analyzed using a computer-based data acquisition system (SuperScope™ software, GW Instruments-Series I; Macintosh IIsi™).

Determination of optimal length-tension relationship

In vitro contractile measurements began with determination of the muscle's optimal stimulation voltage and muscle fiber length required for peak isometric tetanic tension development. To determine the optimal stimulation voltage for maximal muscle force production, the following protocol was performed. Following a 15 minute equilibration period, the muscle strip was stimulated using a Grass model S48 stimulator (modified to deliver 0.5-0.6 amps) (Grass Medical Instruments). Impulses were delivered via field stimulation by two suspended platinum-plate electrodes oriented parallel to the muscle strip along its entire length using monophasic pulses of 2 milliseconds (msec) duration and 50 Hertz (Hz); stimulus intensity was progressively increased until maximal isometric twitch tension was obtained. Supramaximal stimulation was then set at 150% of this value (mean= ~140 volts (V)). We have demonstrated in pilot experiments that this method of stimulation results in maximal muscle stimulation when compared to direct muscle stimulation using stainless steel electrodes.

After determination of the appropriate stimulation voltage, muscle length was adjusted by micromanipulation to its optimum contractile length (L_0) (defined as the muscle fiber length at which maximal active twitch tension is developed, single 0.2 msec pulse). This was accomplished by systematically adjusting the length of the muscle using a micrometer while evoking single

twitch contractions. Thereafter, all contractile measurements were obtained at L_0 .

We chose to measure L_0 using isometric twitches rather than tetanic contractions because twitches are energetically less demanding than tetanic contractions. More importantly, pilot experiments in our lab have shown that L_0 determined using isometric twitches is identical to L_0 determined by tetanic contractions (unpublished observations).

Following completion of contractile measurements, muscle fiber length and strip width were measured at L_0 by microcalipers. The muscle strip was then trimmed free of bone and connective tissue, blotted dry, and weighed.

Measurement of Isometric Twitch Properties

Three twitch stimulations at L_0 (induced by single pulse stimuli, 0.2 msec pulse) were recorded, and the mean values used to determine peak twitch tension (P_t), contraction time (CT), maximal rate of tension development (dP/dt); time to peak twitch tension (TPT; i.e. time required to reach peak force), and one-half relaxation time ($RT_{1/2}$; time required for force to decrease from maximum to one-half maximum).

Measurement of Isometric Tetanic Properties

Measurement of peak isometric tetanic tension

Peak isometric tetanic specific tension (specific P_0) was determined by application of a supramaximal (140V) stimulus train of 50 Hz and one second duration. Each tetanic contraction was separated by a three minute recovery period. Maximal force generation, during both twitch and tetanic contractions

was normalized to cross sectional area (CSA) and expressed as newtons (N)/cm². CSA was estimated on the basis of muscle area (cm²) where muscle area = wet muscle weight (g)/fiber length (cm) × muscle density (g/cm³), where muscle density = 1.056 g/cm³ [89]. In addition, to correct for potential alterations in noncontractile material, maximal force generation was normalized to myofibrillar protein content (CSA) and expressed as N per CSA of strip myofibrillar protein for each animal [90].

Force-velocity relationship

The isotonic force-velocity relationship was determined by the assessment of shortening velocities at 10-12 isotonic loads (100 msec trains of 2 msec pulses at 50Hz) over the range of approximately 10-90% of specific P₀. Force production and shortening velocity data were fitted to the Hill equation [91, 92] with the least-squares technique used to determine the line of best fit. This involves an iterative regression routine in which the computer program (Kaleidograph™, Abelbeck Software) identifies the most appropriate equation for the force-velocity data by determining which values of *a* and *b* provide the smallest sum of squares between the measured and predicted velocities. The maximal velocity of unloaded shortening (V_{max}) was then determined by solving for velocity when force equals zero.

Force-frequency relationship

Response of the diaphragm muscle strip to increasing stimulus frequency (force/frequency relationship) was assessed at L₀ by the application of 10, 20, 30, 40, 45, 50, 60, 80, 100, 150 Hz pulses applied in one second trains. A one minute time period elapsed between contractions.

In vitro Contractile Properties in Costal Diaphragm Single Fibers

Isolated Fiber Preparation

Fiber bundles (5-10 fibers/bundle) were dissected from the intact costal diaphragm section and immediately placed in cold relaxing solution. In order to study the intrinsic contractile apparatus, bundles were chemically permeabilized in a relaxing solution containing 7 mM EDTA, 1.0 mM free Mg^{2+} , 4.38 mM ATP, 14.5 mM creatine phosphate, 20 mM imidazole, and sufficient KCl to adjust ionic strength to 180 mM. This procedure disrupts both the sarcolemma and sarcoplasmic reticulum thereby allowing contractures to be induced by free Ca^{2+} . Following permeabilization, a single fiber was isolated from the fiber bundle and horizontally attached to a stainless steel wire connected to an isometric force transducer (model 400 A; Cambridge Technologies) and length controller (World Precision Instruments). A plexiglass block containing multiple wells (~400 μ l/well) was utilized to hold the relaxing solution as well as various activating solutions (pCa = 4.5 - 8.5) in which the suspended fiber was introduced. The block was manually positioned such that the fiber was submerged within the meniscus of the desired solution. Sarcomere length was determined by directing the beam of a helium-neon laser (Spectra-Physics) through the isolated fiber and observing the primary diffraction pattern in which the distance between the primary diffraction lines is inversely proportional to sarcomere length. Laser calibration was performed by directing the light beam through a grid of known dimensions on a microscope slide. Sarcomere length was set and maintained at ~2.5 μ m (~120% slack length) throughout the force measurements. After completion of an experiment, fiber diameter was measured during a brief exposure to air using a photograph taken from a

video camera interfaced to a fixed lens microscope. The average CSA value was calculated using a calibrated microscope eyepiece micrometer photograph at multiple sites along the length of the fiber. Fiber CSA was calculated assuming a cylindrical fiber shape ($A=\pi r^2$) (where A=fiber area; π =circumference/diameter=3.1416; r=radius) [93].

Maximal Calcium Activated Force and Calcium Sensitivity

Maximal Ca^{2+} -activated force and Ca^{2+} sensitivity were examined by exposing the skinned fiber to various activating solutions until a peak force was attained. Activating solutions were prepared in an identical manner as the relaxing solution except that CaSO_4 was added to obtain pCa (-log free $[\text{Ca}^{2+}]$) levels of 4.5 - 8.5. The amount of CaSO_4 added to obtain each pCa was calculated using the apparent stability constants (adjusted for ionic strength, pH, and T°) and the computer program of Schoenmakers and colleagues [94].

The following protocol was used in the assessment of single fiber contractility and calcium sensitivity. Initially, a maximal contracture was induced (pCa=4.5) followed by four to five submaximal contractures, and finally a second maximal contracture (pCa=4.5). Fibers were allowed to fully relax (in relaxing solution) prior to subsequent activation. The mean force produced during contracture at pCa=4.5 was taken as F_0 . In the course of determining the force-pCa relationship for each fiber, F_0 was determined 2-3 times. If the F_0 values for an individual fiber varied by more than 10%, the data were discarded.

For all experiments, isometric single fiber force production was displayed and recorded by a computer-driven data acquisition system utilizing software

(SuperScope II™ software, GW Instruments-Series II) specifically designed for isolated single fiber contractile measurements.

In addition, force-free Ca^{2+} curves were constructed by fitting the raw data from each fiber (using least squares nonlinear regression) to a modified form of the Hill equation [89]:

$$F_o = [\text{Ca}^{2+}]^N \cdot ([\text{Ca}^{2+}]^N /_{50} + [\text{Ca}^{2+}]^N)^{-1}$$

Dependent variables included: F_o (normalized force; mN), specific F_o ($\text{mN} \cdot \text{mm}^{-2}$), $[\text{Ca}^{2+}]_{50}$ (the Ca^{2+} concentration that evokes 50% of F_o), and the Hill coefficient, N (which is a measure of the slope of the force-pCa relationship).

Biochemical Assays

Myofibrillar Isolation and Protein Determination

Purified myofibrils from the costal diaphragm were obtained using the technique described by Solaro et al. [95] and modified by Caiozzo et al. [5]. Briefly, diaphragmatic muscle samples were cleaned of connective tissue and scissor minced. Muscle samples (100 mg) were then homogenized using a glass on glass homogenizer in 4.0 ml of sucrose buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, 20 mM Tris, pH = 6.8) and centrifuged for 15 minutes at $2500 \times g$. The supernatant was discarded (being careful to leave the myofibrillar pellet intact) and the remaining pellet suspended in 4.0 ml of a KCl buffer containing Triton X-100 (175 mM KCl, 0.5% Triton X-100, 20 mM Tris, pH = 6.8) to eliminate membrane ATPase components. This process was

repeated a final time in 4.0 ml of a KCl buffer (175 mM KCl, 20 mM Tris, pH = 7.0) yielding a purified myofibrillar protein pellet for quantitative assessment of myofibrillar protein concentration determined via the Biuret technique {96}.

Calcium-Activated Myofibrillar ATPase Activity

Immediately following isolation and the assessment of myofibrillar protein concentration, a 0.1 ml sample of purified myofibrils were assayed for myofibrillar ATPase activity using the technique described by Caiozzo et al. {5}. Purified myofibrillar protein were incubated at 25°C in the presence of a maximally activating concentration of Ca^{2+} (pCa=4.0); the reaction was initiated by the addition of 10.0 mM ATP to the reaction medium. ATP hydrolysis was terminated after two minutes by the addition of 1.0 ml of cold 50% trichloroacetic acid. Protein precipitate was then removed from the reaction medium by centrifugation and the concentration of inorganic phosphate in the supernatant determined by the methods of Fiske and Subbarow {97}.

Water Content

Total water content of the costal diaphragm was determined by a freeze drying technique incorporating a negative pressure vacuum pump (-1.0 mm Hg). Frozen samples were placed in the vacuum chamber and freeze-dried for 24 hours prior to determining dry substance mass. True dry mass assessment was confirmed by the measurement of identical weights for each sample following an additional 24 hour period in the vacuum chamber. Total muscle

water content was calculated from the difference between the wet weight of the diaphragm specimen and the dry substance mass of the same sample.

Connective Tissue

Muscle samples were analyzed for total protein and connective tissue concentrations using the technique described by Segal et al. [98]. Briefly, muscle samples were homogenized in 0.9% NaCl and allowed to stand 24 hours in 0.05 M NaOH to solubilize nonconnective tissue. Total muscle protein concentration was measured from a sample of the intact digest. The remaining NaOH digest was centrifuged for 15 min at 4000 × g to sediment collagenous protein. The protein concentration of the supernatant was measured and the connective tissue protein concentration calculated as the difference between protein concentration of the intact digest and that of the digest supernatant. This technique has been shown to be a sensitive measure of muscle connective tissue [99, 100].

Costal diaphragmatic myofibrillar protein, connective tissue, and water content were calculated by multiplying the appropriate concentration by the total costal wet weight for each animal.

Analysis of Myosin Isoforms by Polyacrylamide Gel Electrophoresis

Electrophoresis involves the separation of protein mixtures due to varying mobility's of the protein molecules in an imposed electric field. Polyacrylamide gel electrophoresis (PAGE) is carried out in a support medium of polymerized acrylamide of varying concentration and thus porosity. The porosity of the gel in turn determines protein mobility by a logarithmic

dependence on the species molecular weight [101]. The electrophoretic method for denatured proteins involves the use of sodium dodecyl sulfate (SDS-PAGE) which is adsorbed to the polypeptide chain. Because of the lack of any native structure, mobility of the species is dependent on molecular weight while excluding any shape considerations [101].

Non-Dissociating Polyacrylamide Gel Electrophoresis

Crude homogenate samples were used to separate ventricular native myosin isoforms by a nondissociating PAGE procedure according to Hoh et al. [87]. Approximately 5 µg of protein were loaded onto 6 cm long tube gels and electrophoresed (constant current of 3 mA/tube) in a modified tube gel apparatus (Fisher Scientific) for 20 hours at 4°C. Sodium pyrophosphate (20mM) buffer (pH=8.8) was recirculated continuously during electrophoresis. Gels were stained with R-250 Coomassie blue (1 hour) and destained for 12 hours using a 30% methanol / 7% acetic acid solution by diffusion. Native myosin were identified using reference native myosin isolated from control muscle.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Identification and Distribution of MHC

Myosin heavy chains were separated using a 8% polyacrylamide gel system first described by Laemmli et al. [102] and later modified by Talmadge and Roy [103]. Briefly, myofibrillar protein samples were diluted in glycerol to a concentration of ~1.0 mg/ml (samples can be stored in this form at -20°C for

up to two months). Glycerinated myofibrillar samples were further diluted to a concentration of ~0.25 mg/ml in sample buffer containing 62.5 mM Tris (pH=6.8), 1.0% SDS, 0.01% bromophenol blue, 15% glycerol, and 5% β -mercaptoethanol and denatured by incubation at ~95°C for five minutes.

Approximately 3.0 μ g of protein was loaded per well onto 22 cm vertical gels (Biorad™ Protean IIxi) composed of 40% glycerol. Samples were electrophoresed for 20-24 hours at ~5°C using a constant voltage required to attain an initial current of 12 milliamps per gel. Gels were fixed and stained in a solution containing R-250 Coomassie blue (1 hour) and destained for 12 hours in a 30% methanol / 7% acetic acid solution by diffusion. MHC's were identified by using a combination of high molecular weight markers and reference MHC's isolated from control muscle.

Isolated costal diaphragm single fibers were dissolved in 10 μ l of sample buffer and incubated at 95°C for five minutes to denature the contractile protein. The entire volume (10 μ l) was then electrophoresed as described above for determination of single fiber MHC isoform content. Due to the small quantity of myofibrillar protein present, diaphragm single fibers were characterized using a silver stain technique described by Switzer et al. [104].

Quantification of Native Myosin and Myosin Heavy Chains

Computerized densitometric image analysis system including a Targa M8 image capture board (Truevision) and Java video analysis software (Jandel Scientific) integrated with a high resolution CCT video camera (Video World, Inc.) were utilized to determine the relative distributions of the various native myosin isoenzymes and MHC isoforms. The region of the gel containing the MHC bands was digitized and the area and intensity of

staining determined in duplicate for each band. To quantify the relative proportions of native myosin and MHC (percent of total myosin pool), the area for each individual band was multiplied by the average intensity (OD) for that band to give an (area x OD) value. Values representing the individual isoforms for a given lane were summated to give a total figure representing 100% of the total myosin present. The percentage of a given isoform or subunit was then determined on the basis of its relative contribution (area x OD) to the total myosin pool. In our laboratory, the coefficient of variation for repeated measurement of relative native myosin or MHC composition of a given sample is <5.0%. As an additional check of protein sample purity, random myofibrillar samples were electrophoresed in the second dimension and subsequently underwent isoelectric focusing to ensure sample purity.

Statistical Analysis

Intergroup (control vs. hypothyroid) comparisons for each dependent variable, with the exception of the single fiber measurements, were made by independent *t*-tests. Single fiber contractile data (e.g. maximum Ca^{2+} -activated specific F_0 , CSA) were analyzed by independent *t*-test comparing type I and type II fibers between groups. To account for alpha level inflation (increased likelihood of type I error) the Bonferroni procedure was used where appropriate. Data (except where noted) are expressed as means \pm SEM. Correlational analysis to determine the relationship between specific biochemical measures and selected contractile parameters of the diaphragm were assessed using Pearson product moment correlation. Significance was established at $p < 0.05$.

CHAPTER 4 RESULTS

Evidence of Hypothyroid State

Thyroid status was assessed utilizing the following variables: 1) free T_3 concentration (pg/ml), 2) resting metabolic rate ($\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), 3) cardiac mass (mg) and 4) native cardiac myosin isoform profile. These findings are summarized in table 1. As evidenced by these data, the combination of surgical thyroidectomy and n-propylthiouracil (PTU) treatment was extremely effective in suppressing circulating levels of free T_3 . Eleven of the thirteen animals in the hypothyroid group had free T_3 levels that were <0.2 pg/ml (below the lowest detectable concentration). For statistical purposes, these animals were assigned free T_3 levels of 0.2 pg/ml. The two remaining animals although having slightly higher free T_3 levels (0.73 and 0.84 pg/ml, respectively), exhibited physiological profiles consistent with hypothyroidism in all other parameters measured and were therefore considered to be in a hypothyroid state. Further, animals in the hypothyroid group had significantly ($p < 0.05$) lower total heart weights (table 1) and a native cardiac isomyosin profile characterized by the predominance of the low ATPase isoform, V_3 (table 1, figure 1). Finally, resting metabolic rates, measured by indirect calorimetry, were depressed 30.6% ($p < 0.05$) in the hypothyroid group compared to controls (table 1). Collectively, these results are consistent with a hypothyroid state in rats [5, 48].

Morphometric Characteristics

Physical characteristics of the animals studied are summarized in table 2. Mean body weights between groups at the initiation of the project were similar, however, following six weeks of experimental treatment, mean body mass of the hypothyroid animals was reduced 15.3% ($p < 0.05$) in comparison to controls.

Similar to body mass, total diaphragmatic mass in the hypothyroid group was significantly lower ($p < 0.05$) than control animals (-5.4%). Further, mean costal diaphragmatic wet weight was 6.0% less ($p < 0.05$) in hypothyroid animals compared to controls. No mass differences were noted in the crural portion of the diaphragm ($p > 0.05$). Finally, in comparison to controls, both costal and crural diaphragm/body mass ratios (table 2) were higher ($p < 0.05$) in the hypothyroid group, indicating that the hypothyroid state had less effect on diaphragmatic muscle mass compared to total body mass.

Muscle Biochemical Characteristics

Table 2 summarizes the measured biochemical variables of the costal diaphragm in both control and hypothyroid animals. Myofibrillar protein concentration (normalized to muscle wet weight (mg/g)) did not differ between groups ($p > 0.05$). Further, no group differences in diaphragmatic water content (dry mass/unit wet mass) was observed. Total diaphragmatic protein content did not differ ($p > 0.05$) between experimental and control groups. In addition, diaphragmatic connective tissue content was unchanged in hypothyroid versus control animals ($p > 0.05$).

Collectively, these observations support the notion that hypothyroidism does not alter contractile and noncontractile proteins or water content in the adult rodent costal diaphragm.

Costal Diaphragm MHC Isoforms and Myofibrillar ATPase Activity

Hypothyroid-induced alterations in costal diaphragm MHC isoform content are summarized in figure 2 and table 3. Six weeks of hypothyroidism produced significant shifts in type IId/x, type IIb, and type I MHC isoform distribution (see figure 3 for a representative gel). Specifically, hypothyroidism produced a 68.2% increase in type I MHC content (table 3 and figures 2) while reducing type IId/x and type IIb isoform content (table 3, figures 2 and 3) by 11.8 and 82.7% respectively. Consistent with a fast to slow shift in MHC distribution, mean costal diaphragmatic myofibrillar ATPase activity in the hypothyroid group was reduced 30.5% ($p<0.05$) compared to controls (table 2).

In vitro Contractile Properties of Costal Diaphragm Strips

Table 4 illustrates the morphometric and contractile characteristics of the *in vitro* costal diaphragm strip for the experimental and control groups. Costal diaphragm strip length at L_0 was greater ($p<0.05$) in control compared to hypothyroid diaphragms. However, no difference existed in cross sectional area (CSA) for the costal diaphragm strips used in the assessment of contractile function ($p>0.05$).

Isometric Twitch Properties

As illustrated in table 4, hypothyroid animals exhibited a marked slowing in several isometric twitch characteristics of the costal diaphragm. Maximal rate of tension development (as measured by the derivative, dP/dt) and time to peak twitch tension (TPT; time required to reach peak isometric force) were significantly increased ($p<0.05$) 27.8% and 15.5% respectively, in diaphragms of hypothyroid animals. Further, one-half relaxation time ($RT_{1/2}$; time required for force to decrease from maximum to one-half maximum) was slowed 64.8% ($p<0.05$) while contraction time (CT) was also significantly prolonged (27.4%; $p<0.05$) in response to hypothyroidism. Finally, the P_t/P_o ratio was significantly increased in hypothyroid diaphragms ($p>0.05$). No group differences existed for peak specific twitch tension (P_t) (see table 4).

Isometric and Isotonic Tetanic Characteristics

Peak isometric tetanic specific tension

Maximal specific tetanic tension expressed per cm^2 of strip CSA was 21.1% lower ($p<0.05$) in the hypothyroid group as compared to controls. When specific tension for each strip was normalized to myofibrillar protein CSA, the resulting specific P_o remained significantly reduced in the hypothyroid animals (-19.2%) when compared to controls (see table 4).

Force-velocity relationship

Figure 4 illustrates the impact of hypothyroidism on the force-velocity relationship of the costal diaphragm. Note that the relationship between muscle force production and speed of shortening in the hypothyroid group is

shifted down and to the left of the control animal data. Maximal shortening velocity of the hypothyroid group was significantly less (~25%) than controls ($p < 0.05$) (table 4). Finally, the a/P_o ratio, which represents the curvature of the force-velocity relationship (where a is a calculated value of the Hill equation and P_o is the measured maximal specific P_o), was significantly altered ($p < 0.05$) by hypothyroidism (table 4).

Examination of the relationship between Ca^{2+} -activated myofibrillar ATPase activities as well as MHC isoform composition and costal diaphragm maximal shortening velocity were performed by linear regression analysis. Figure 5 shows the relationship between myofibrillar ATPase activity and costal diaphragmatic V_{max} . Costal diaphragms having the lowest myofibrillar ATPase activities (hypothyroid animals) demonstrated reduced V_{max} values (mean \pm SEM; 3.83 L/sec \pm 0.11) while control diaphragms exhibited higher myofibrillar ATPase activities and thus an elevated mean V_{max} (5.11 L/sec \pm 0.14) (table 4). The coefficient of determination (r^2) for the relationship between myofibrillar ATPase activity and V_{max} was 0.64 ($r = 0.80$).

Further, figure 6 indicates the strong positive relationship between type IIb MHC distribution and V_{max} , ($r = 0.78$), while figure 7 illustrates the strong inverse relationship between type I MHC content and V_{max} , ($r = -0.83$).

Force-frequency relationship

The force-frequency curves for the control and hypothyroid groups are shown in figure 8. Mean (\pm SEM) group data for diaphragmatic specific force production in both groups were plotted against stimulation frequencies ranging from 10 to 150 Hz. Tetanic specific force production reached a plateau at 50 Hz in both control and hypothyroid animals. Further, note that specific

force production was greater ($p < 0.05$) in control compared to hypothyroid costal strips at all stimulation frequencies above 30 Hz.

In vitro Contractile Properties in Costal Diaphragm Single Fibers

Table 5 presents mean (\pm SEM) CSA, maximal Ca^{2+} -activated force (F_0), and maximal specific F_0 of isolated costal diaphragmatic skinned single fibers (types I and II) obtained from control and hypothyroid animals.

Cross Sectional Area

Isolated single fibers from nine control (34 individual costal diaphragm fibers) and eleven hypothyroid animals (36 individual costal diaphragm fibers) were measured for fiber diameter at 120% of optimal length (standard sarcomere length of $2.5\mu\text{m}$) (table 5). The mean diameter of the control fibers was $47.9 \pm 12.2\mu\text{m}$, while hypothyroid fibers were $42.1 \pm 16.5\mu\text{m}$ ($p > 0.05$).

Assuming a cylindrical fiber shape, this corresponds to a calculated mean cross-sectional area of $1737.9 \pm 174.7 \mu\text{m}^2$ for control type I fibers and $1487.4 \pm 124.7 \mu\text{m}^2$ for control type II fibers ($p > 0.05$). CSA of type I hypothyroid fibers did not differ from type I control fibers, while CSA of type II hypothyroid fibers were also similar to control values (see table 5).

Maximal Calcium Activated Specific Force

A specific tension value (F_0) was calculated for each fiber in which a diameter measurement, MHC phenotype, and maximal Ca^{2+} -activated absolute force was obtained. Fibers were classified as type I or type II based on

their MHC profile (see representative gel, figure 9). Due to a small sample size, all fibers exhibiting type II MHC phenotype (type IIa, type IIb, and type IIc) were analyzed together. Mean specific F_0 of type I control fibers were ($n=9$ animals; 19 isolated single fibers) similar to type I ($n=11$; 24 isolated single fibers) hypothyroid fibers. Further, control type II fibers ($n=11$; 15 isolated single fibers) and hypothyroid type II fibers ($n=11$; 12 isolated single fibers) did not differ in specific force production. This finding is important since it does not support the hypothesis that *in vitro* diaphragmatic single fibers from control and hypothyroid animals differ in maximal Ca^{2+} -activated specific force development.

Figure 10 compares the specific F_0 of hypothyroid type I and type II single fibers to the specific F_0 of hypothyroid *in vitro* diaphragm strips (expressed as a percentage of the corresponding control mean value). Again, note that the specific force deficit present in the hypothyroid strip (when compared to control) is not present in the hypothyroid single fiber preparation.

Force-pCa Relationship

Twenty-one control (6 animals) and nine hypothyroid fibers (5 animals) underwent graded isometric contractions at specific free Ca^{2+} concentrations (pCa range of 8.5 - 4.5). These data were then fitted to a modified Hill equation {Hill, 1938} for the characterization of the force/pCa relationship for each individual fiber. The relationship between group mean force production and free Ca^{2+} concentration (pCa) are summarized in figure 11. The diagram clearly indicates that hypothyroid fibers exhibit a leftward shift in the force/pCa relationship; thus indicating an increased Ca^{2+} sensitivity of those fibers. This finding is further supported by a significant reduction in the $[Ca^{2+}]_{50}$, (the Ca^{2+}

concentration that evokes 50% of F_0), of the hypothyroid fibers, again suggesting an increased Ca^{2+} sensitivity of those fibers. Although statistical analyses were performed on the $[\text{Ca}^{2+}]_{50}$ values, mean values for pCa were also calculated and are presented in table 5. Finally, the Hill coefficient, N , (a measure of curvature of the force/pCa relationship) was not significantly different ($p>0.05$) between control and hypothyroid fibers. Electrophoretic assessment of single fiber MHC phenotype revealed that ~78% (7 of 9) of the fibers in the hypothyroid group expressed the slow, type I MHC isoform. Conversely, only ~24% (5 of 21) single fibers obtained from control animals expressed the slow heavy chain isoform. This is important since it is well known that slow, type I single fibers exhibit an increased Ca^{2+} sensitivity when compared to fast, type II fibers [105].

Table 1. Indexes of thyroid status in adult, euthyroid {control} and hypothyroid female, Sprague-Dawley rats.

<u>Parameter</u>	<u>Control</u>	<u>Hypothyroid</u>	<u>Δ, {%</u>
<i>n</i>	12	13	
Free T ₃ concentration {pg/ml}	1.39±0.07	0.29±0.06**	-79.1
Resting oxygen uptake {ml•kg ⁻¹ •min ⁻¹ }	30.97±0.87	21.5±0.66**	-30.6
Total cardiac mass {mg}	996.3±13.7	675.7±11.9**	-32.2
Native cardiac myosin isoforms {% of total myosin pool}			
V1	10.6	0**	
V2	17.9	0**	
V3	71.5	100**	

Values are means±SEM.

n, sample size; T₃, triiodothyronine; pg, picogram.

** *p* < 0.05, significantly different from control.

Table 2. Morphological and biochemical characteristics of adult, control and hypothyroid female, Sprague-Dawley rats.

<u>Parameter</u>	<u>Control</u>	<u>Hypothyroid</u>	<u>Δ, (%)</u>
<u>Morphological</u>			
<i>n</i>	12	13	
Initial body mass {g}	271.9 \pm 2.6	270.2 \pm 3.1	-0.6
Final body mass {g}	304.4 \pm 4.9	257.8 \pm 5.0**	-15.3
Costal diaphragm mass {mg}	634.1 \pm 15.2	595.9 \pm 7.4**	-6.0
Costal/body mass ratio {mg/g}	2.08 \pm 0.04	2.32 \pm 0.04**	11.5
Crural diaphragm mass {mg}	306.5 \pm 7.1	294.0 \pm 5.5	-4.1
Crural/body mass ratio {mg/g}	1.01 \pm 0.02	1.14 \pm 0.03**	12.9
Total diaphragm mass {mg}	940.7 \pm 20.5	889.9 \pm 10.4**	-5.4
<u>Biochemical</u>			
<i>n</i>	12	13	
Costal myofibrillar protein content {mg/g wet weight}	124.3 \pm 8.9	120.9 \pm 9.7	-2.7
Costal myofibrillar ATPase activity {nM P _i •mg ⁻¹ •min ⁻¹ }	469.9 \pm 14.5	326.4 \pm 19.8**	-30.5
Costal dry mass {mg/g wet weight}	298.2 \pm 4.2	287.7 \pm 5.4	-3.5
Costal connective tissue {mg/g wet weight}	30.6 \pm 4.1	32.2 \pm 3.4	5.2

Values are means \pm SEM. *n*, sample size; P_i, inorganic phosphate.

** *p* < 0.05, significantly different from control.

Table 3. Myosin heavy chain distribution of costal diaphragm from adult, control and hypothyroid female, Sprague-Dawley rats.

<u>Group</u>	<u>n</u>	<u>Myosin heavy chain isoform</u>			
		<u>Type I</u>	<u>Type IIa</u>	<u>Type IIc/x</u>	<u>Type IIb</u>
Control	12	24.5±2.7	19.9±3.0	40.0±5.2	15.6±6.6
Hypothyroid	13	41.2±2.5**	20.8±2.5	35.3±2.1**	2.7±1.5**
Δ , {%}		68.2	4.5	-11.8	-82.7

Values are means±SD expressed as a percentage of the total myosin pool.
n, sample size.

** p < 0.05, significantly different from control.

Table 4. *In vitro* costal diaphragm strip contractile characteristics in adult, control and hypothyroid female, Sprague-Dawley rats.

Parameter	Control	Hypothyroid	Δ , {%}
<i>n</i>	12	13	
<u>Morphometric</u>			
L_o {mm}	21.6±0.48	18.2±0.31**	-15.7
CSA {cm ² }	0.01937±0.00086	0.01976±0.000066	2.0
<u>Tetanic properties</u>			
P_o {N•cm ⁻² }	24.36±0.41	19.22±0.46**	-21.1
P_o {N•[MP]cm ⁻² }	197.6±5.56	159.6±5.09**	-19.2
V_{max} {L•sec ⁻¹ }	5.11±0.14	3.83±0.11**	-25.0
a/P_o	0.18±0.009	0.26±0.019**	44.4
<u>Twitch properties</u>			
P_t {N•cm ⁻² }	6.5±0.28	5.9±0.24	-9.2
TPT {msec}	57.9±12.2	66.9±12.7**	15.5
dP/dt {N•cm ⁻² •msec}	0.18±0.007	0.13±0.007**	-27.8
RT _{1/2} {msec}	50.3±13.1	82.9±14.1**	64.8
CT {msec}	135.9±16.6	173.1±12.6**	27.4
P_t/P_o ratio {N•cm ⁻² }	0.267±0.012	0.310±0.015**	16.1

Values are means±SEM.

n, sample size; L_o , optimal muscle fiber length; CSA, cross sectional area; P_o , maximal specific isometric tetanic tension; N, newtons; {N•[MP]cm⁻²}, specific force normalized to strip myofibrillar protein CSA; V_{max} , maximal shortening velocity extrapolated from the Hill equation; a/P_o , curvature of force-velocity relationship where *a* is a constant derived from the Hill equation; P_t , peak isometric twitch specific tension; TPT, time to peak tension; RT_{1/2}, one-half relaxation time; dP/dt, maximal rate of specific tension development; CT, contraction time.

** $p < 0.05$, significantly different from control.

Table 5. Contractile characteristics of costal diaphragm skinned single fibers based on MHC isoform distribution from adult, control and hypothyroid female, Sprague-Dawley rats.

	<u>Control</u>	<u>Hypothyroid</u>	<u>Δ, {%</u>
<u>Type I MHC</u>	<u>n=9</u> <u>SF=19</u>	<u>n=11</u> <u>SF=24</u>	
CSA (μm^2)	1737.9 \pm 174.7	1487.4 \pm 124.7	-14.4
F ₀ (mN)	0.155 \pm 0.017	0.142 \pm 0.012	-8.4
Specific F ₀ (mN \cdot mm ⁻²)	105.4 \pm 6.4	105.6 \pm 7.5	0.2
<u>Type II MHC</u>	<u>n=8</u> <u>SF=15</u>	<u>n=7</u> <u>SF=12</u>	
CSA (μm^2)	1522.7 \pm 211.1	1288.5 \pm 123.7	-15.4
F ₀ (mN)	0.158 \pm 0.009	0.143 \pm 0.018	-9.5
Specific F ₀ (mN \cdot mm ⁻²)	118.1 \pm 13.7	121.5 \pm 7.3	2.9
<u>Calcium sensitivity</u>	<u>n =6</u> <u>SF=21</u>	<u>n =5</u> <u>SF=9</u>	
[Ca ²⁺] ₅₀ (μM)	1.79 \pm 0.73	0.65 \pm 0.06**	-63.7
pCa ₅₀	5.75	6.19	
N	1.34 \pm 0.11	1.49 \pm 0.18	11.1

Values are means \pm SEM.

n, animal sample size; MHC, myosin heavy chain; SF, number of isolated single fibers measured *in vitro*; CSA, cross sectional area; F₀, maximal Ca²⁺-activated force; mN, millinewtons; [Ca²⁺]₅₀, concentration of Ca²⁺ required to evoke 50% of F₀; pCa₅₀, -log [Ca²⁺]₅₀; N, Hill coefficient- indicates curvature of force-pCa relationship.

** *p* < 0.05, significantly different from control.



Figure 1. Electrophoretic separation of cardiac native myosin isoforms from control and hypothyroid animals. Note all 3 cardiac isoforms are expressed in the control heart, with V₁ the predominant isoform. Alternatively, in the hypothyroid state, V₁ and V₂ myosin isoform expression is completely repressed.

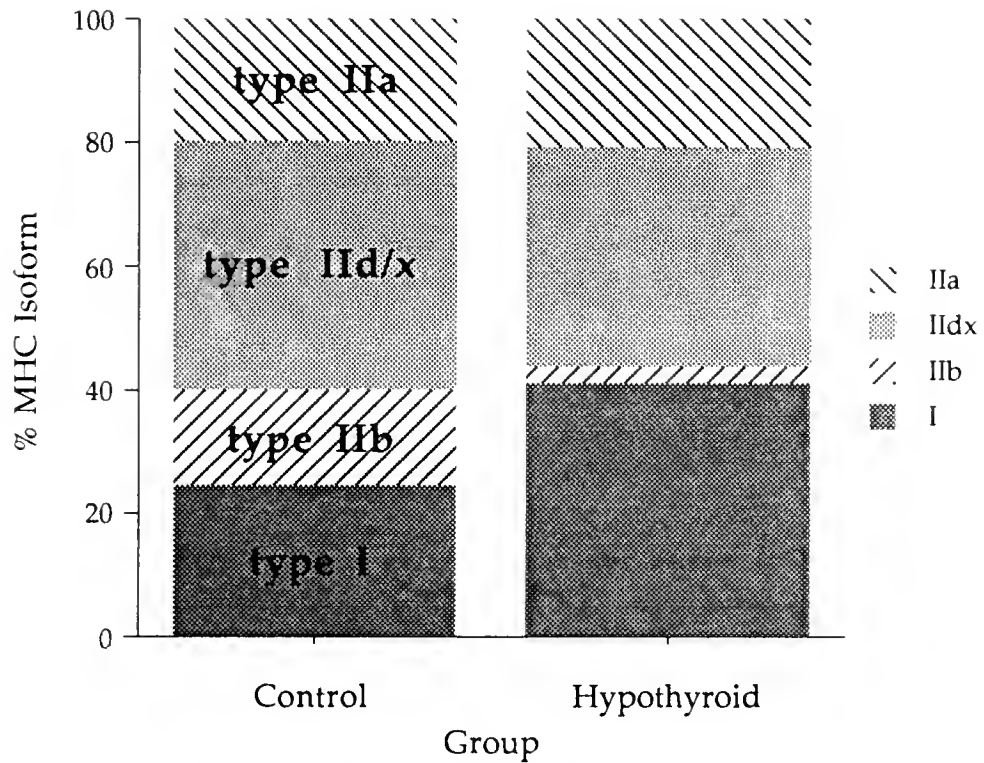


Figure 2. Comparison of costal diaphragm myosin heavy chain isoform profiles between control and hypothyroid animals. Note the significant reduction in type IIx and IIb MHC isoform content, while type I MHC distribution is significantly increased.

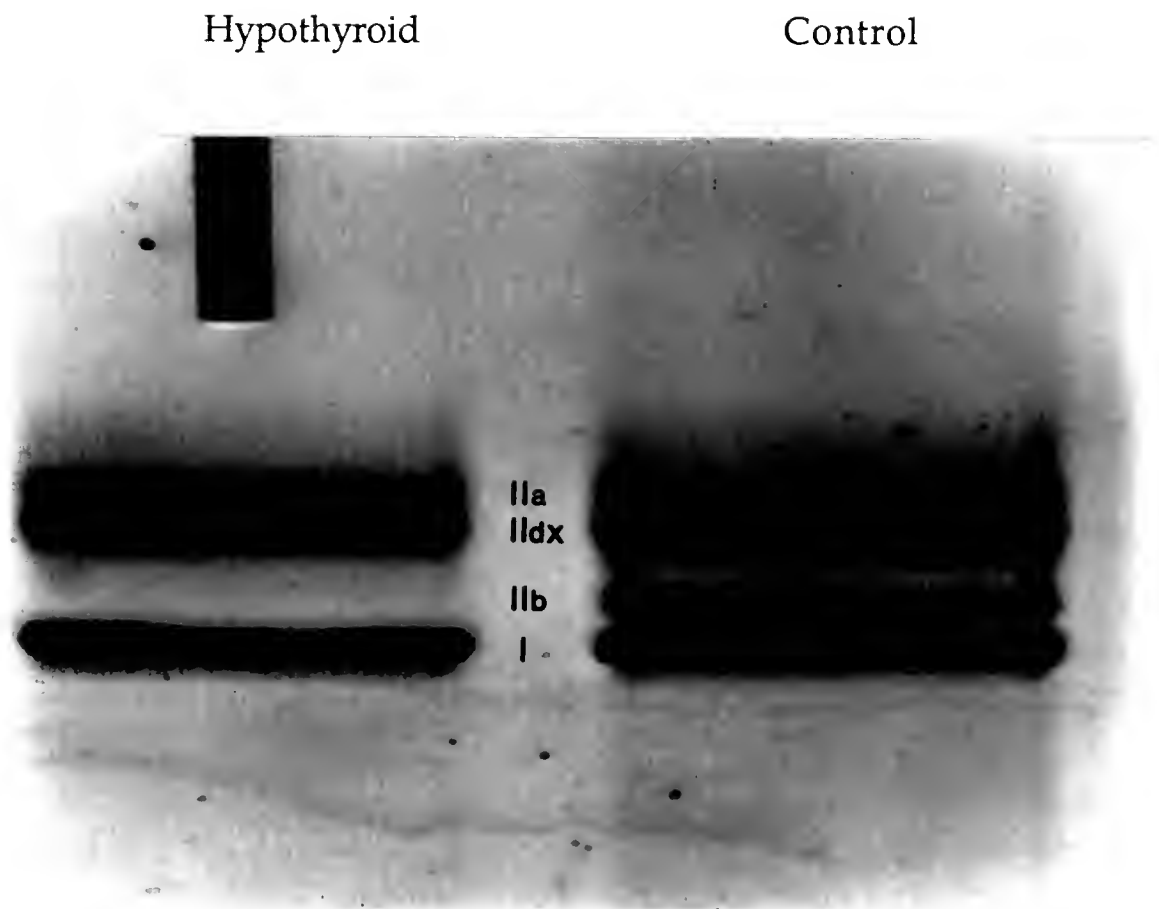


Figure 3. Photograph of electrophoretic (SDS-PAGE) separation of costal diaphragm myosin heavy chain (MHC) isoforms from control and hypothyroid animals. Note the significant reduction in type IIb MHC in the hypothyroid animal.

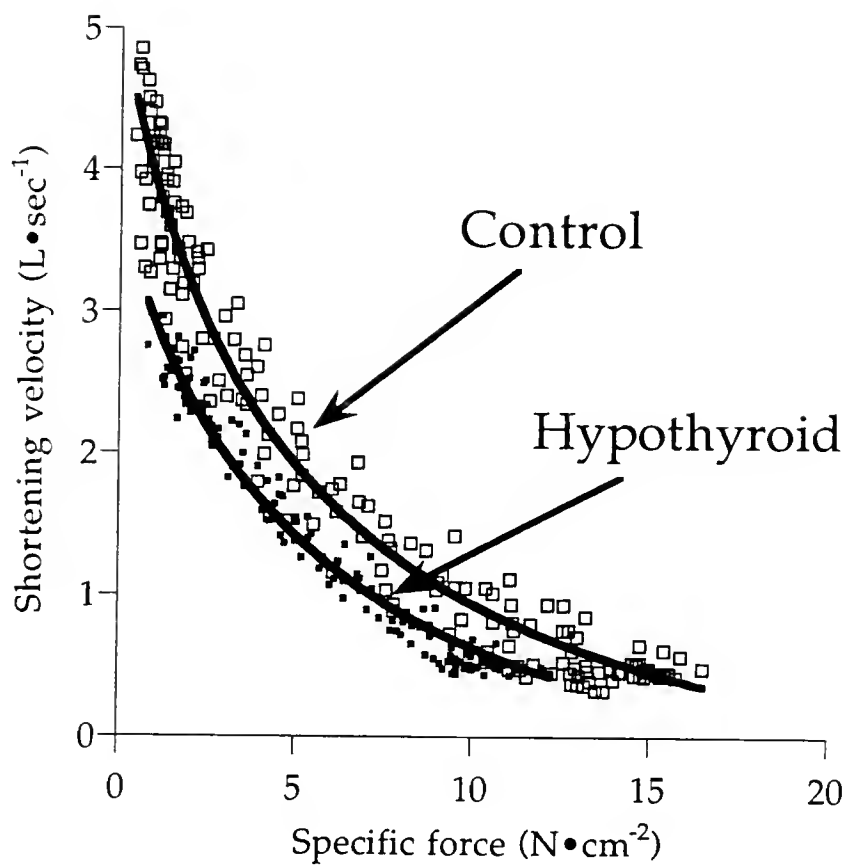


Figure 4. Force-velocity relationships for control and hypothyroid *in vitro* costal diaphragm strips. To account for differences in muscle fiber length, velocity of shortening is expressed as muscle lengths per second ($L \cdot \text{sec}^{-1}$).

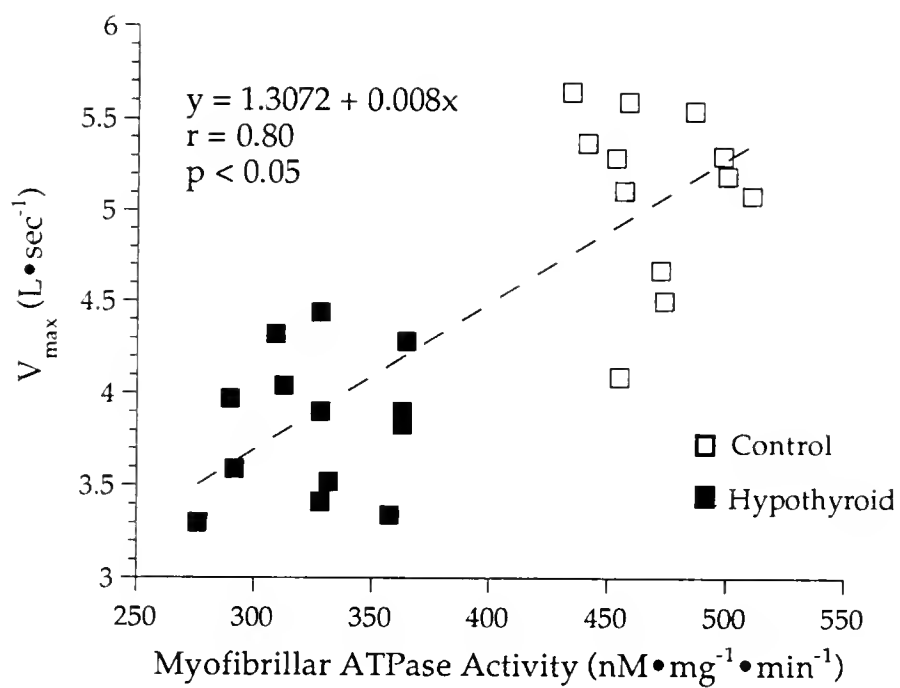


Figure 5. Correlational analysis of the relationship between costal diaphragmatic maximal shortening velocity (V_{\max}) and myofibrillar ATPase activity.

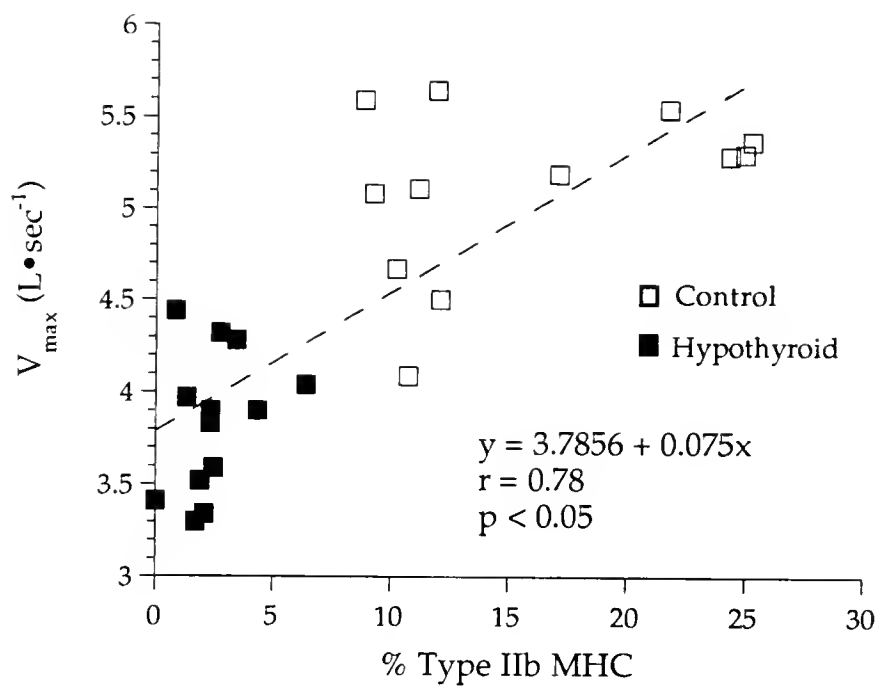


Figure 6. Correlational analysis of the relationship between costal diaphragmatic maximal shortening velocity (V_{\max}) and type IIb myosin heavy chain content.

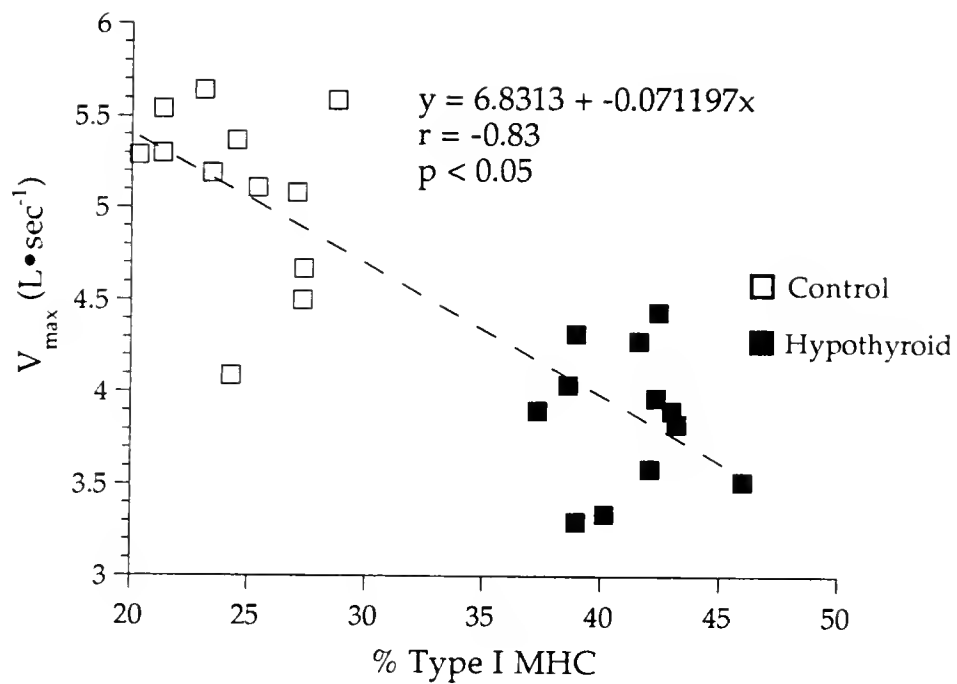


Figure 7. Correlational analysis of the relationship between costal diaphragmatic maximal shortening velocity (V_{\max}) and type I myosin heavy chain content.

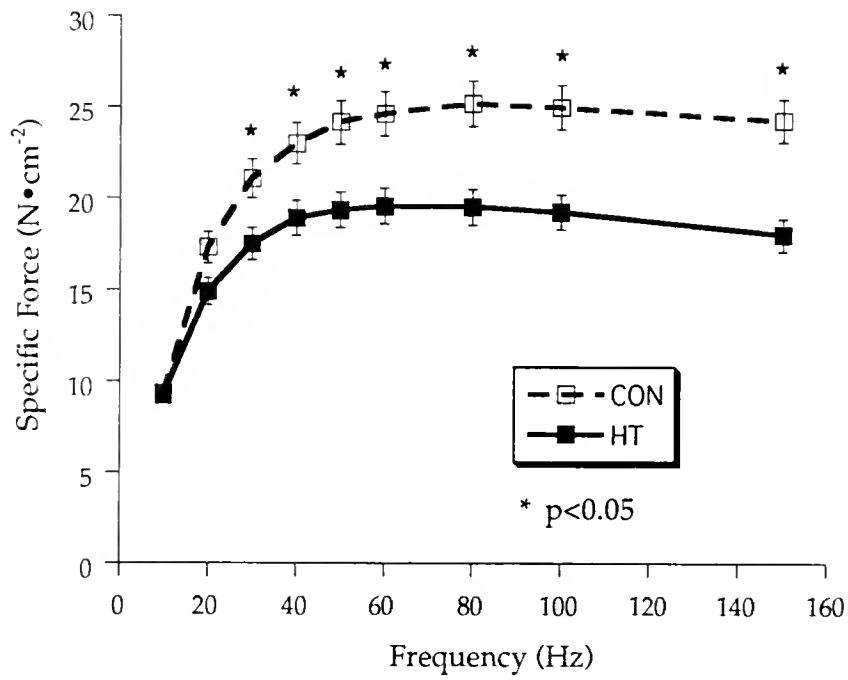
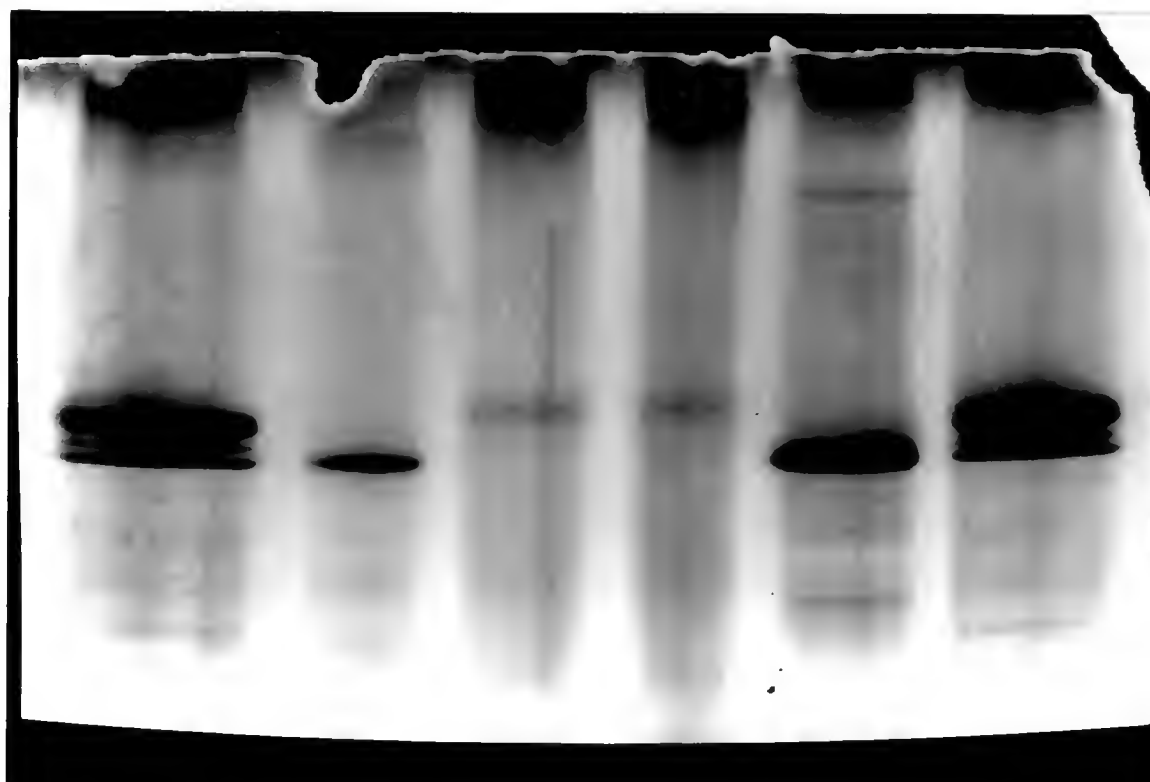


Figure 8. Force-frequency relationship of *in vitro* costal diaphragms strips from control (CON) and hypothyroid (HT) animals.



Lane 1 2 3 4 5 6

Figure 9. Representative photograph of electrophoretic (SDS-PAGE) separation of myosin heavy chain isoforms obtained from isolated costal diaphragm single fibers.

Lane 1. Costal diaphragm fiber bundle showing expression of all 4 MHC isoforms present in adult rodent.

Lane 2. Single fiber expressing type I MHC

Lane 3. Single fiber expressing type IIa MHC

Lane 4. Single fiber co-expressing type IIa and I MHC

Lane 5. Single fiber expressing type I MHC

Lane 6. Costal diaphragm fiber bundle showing expression of all 4 MHC isoforms present in adult rodent.

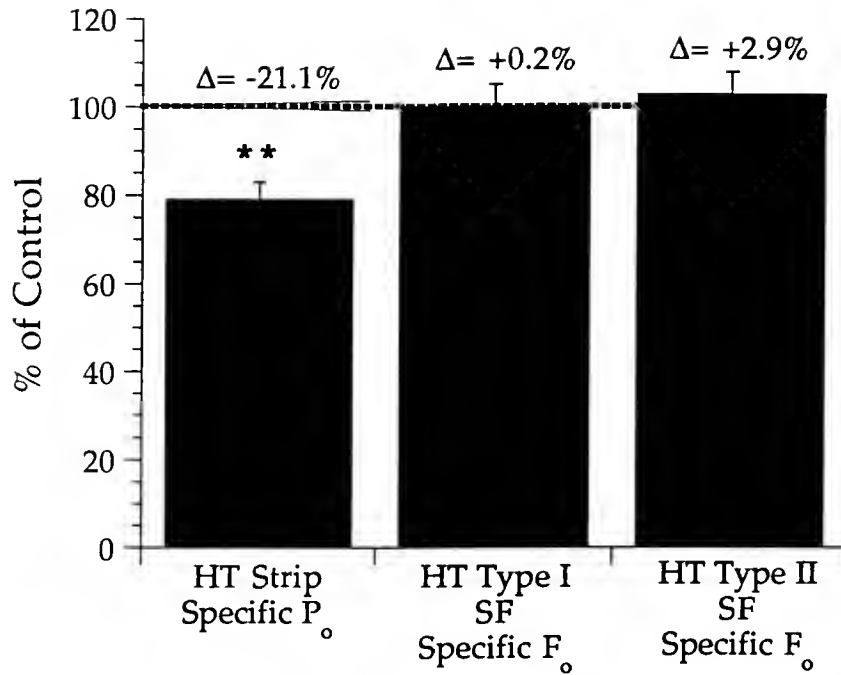


Figure 10. Comparison of *in vitro* maximal specific tensions of costal diaphragm strips (specific P_o ; $N \cdot cm^{-2}$) and maximal Ca^{2+} -activated skinned single fibers (SF; specific F_o ; $mN \cdot mm^{-2}$) in control (CON) and hypothyroid (HT) animals. Values for the hypothyroid group are presented as a percentage of control values. ** $p < 0.05$

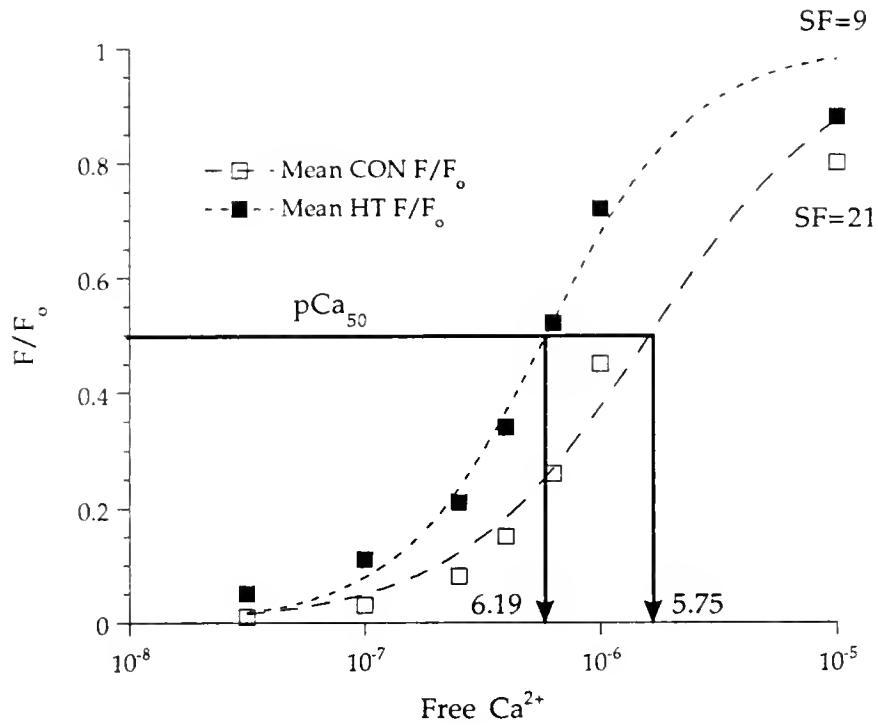


Figure 11. Force-pCa relationship between costal diaphragm isolated single fibers from control (CON) and hypothyroid (HT) animals. Single fibers (SF) denotes the number of isolated myofibrils from each group used for assessment of force-pCa curve.

CHAPTER 5 DISCUSSION

Overview and Principal Findings

This study tested two hypotheses specific to the hypothyroid-induced contractile dysfunction observed in the costal diaphragm. First, it was postulated that the hypothyroid-induced decrease in diaphragmatic specific tension is due to an intrinsic alteration in the force generating capacity of the isolated myofibril. Secondly, the hypothesis that the reduction in maximal shortening velocity of the hypothyroid diaphragm is strongly correlated to a reduced myofibrillar ATPase activity and a fast to slow shift in MHC isoform content was tested.

Results indicate that the hypothyroid-induced reduction in diaphragmatic specific tension is unrelated to intrinsic changes in myofibril maximal force generation and is not due to alterations in myofibrillar protein concentration. Therefore by elimination, these findings suggest some step of excitation-contraction coupling (E-C coupling) as the primary cause of the *in vitro* costal diaphragm specific force deficit.

Finally, the second hypothesis is supported by the strong correlational relationships between myofibrillar ATPase activity, type I and IIb MHC isoforms, and reduced *in vitro* maximal shortening velocities in hypothyroid costal diaphragm strips.

In vitro Isometric Specific Force in Costal Diaphragm Strips and Single Fibers

While several investigators have demonstrated a reduction in specific force generation in hypothyroid hindlimb skeletal muscle [5, 33], this report is the first to document a reduced *in vitro* maximal isometric specific force in the costal diaphragm of hypothyroid animals. These findings are consistent with investigators who have observed clinical impairments in tetanic force development in locomotor [25, 30, 35] and respiratory [1-4] muscle of hypothyroid patients.

Theoretically, diaphragmatic specific P_0 is determined by the following factors: 1) the number of cross-bridges (myofibrils) in parallel per muscle CSA, 2) maximal force generation per crossbridge, 3) myofilament activation by excitation-contraction (E-C) coupling or 4) some combination thereof. In the present study, no hypothyroid-induced changes were seen in myofibrillar protein (cross-bridge) concentration, connective tissue concentration, or water content. Collectively, these data suggest that hypothyroidism does not result in a dilution in contractile protein concentration (cross-bridges) which could potentially reduce muscle force generation.

The use of animals exhibiting pronounced hypothyroidism improved the ability to test the hypothesis that alterations in the intrinsic nature of the hypothyroid myofibril is responsible for the observed deficit in diaphragmatic specific P_0 . The use of the chemically skinned single fiber preparation allowed the determination of intrinsic differences in the force generating ability and sensitivity of the contractile apparatus to Ca^{2+} in hypothyroid fibers. By comparing the specific P_0 of muscle strips with F_0 from single fibers (between control and hypothyroid animals), we were able to indirectly determine if the deficit in P_0 of hypothyroid diaphragm strips is intrinsic to the contractile

apparatus (decreased P_o and F_o) or related to some step in E-C coupling (decreased P_o with no change in F_o). This reasoning is based on the idea that if the percent deficit in specific F_o of hypothyroid diaphragm fibers is substantially smaller than the percent deficit for specific P_o (hypothyroid costal diaphragm strips), the majority of the force decrement in the *in vitro* muscle strip is likely due to an impairment in E-C coupling (Ca^{2+} handling) to the myofibrils during tetanic tension development. This explanation seems plausible given that E-C coupling is by-passed in the skinned fiber preparation and that activation of the myofibrils is directly controlled by Ca^{2+} concentration in the medium surrounding the fiber.

To our knowledge, this is the first study to compare contractile properties in type I and II diaphragmatic single fibers from control and hypothyroid animals (table 5). Specific F_o and CSA measurements obtained in the present study are in agreement with published values for both slow and fast fibers of the adult rodent diaphragm [59]. Due to small sample sizes, analyzing single fiber specific F_o based on individual fast MHC isoform content (types IIa, IIc/x, and IIb) was not statistically possible. Therefore, the fast MHC isoforms (types IIa, IIc/x, and IIb) in the hypothyroid fiber group were combined and compared to the pooled control type II fiber sample. Such an approach should provide valid information since it is possible to compare fast and slow single fibers between control and hypothyroid groups.

Utilizing the single fiber preparation, we tested the hypothesis that the specific diaphragmatic force deficit in hypothyroid animals was due to an intrinsic reduction in the force generating capacity of the contractile apparatus (maximal force generation per cross-bridge). Maximal Ca^{2+} -activated specific force measurements obtained from isolated, (type I and II) single fibers indicated no difference between control and hypothyroid animals in the

ability of the contractile apparatus to generate tension (table 5). These data indicate that hypothyroidism does not alter the intrinsic nature of the individual crossbridges to develop maximal force (specific F_0). This finding, in conjunction with the lack of alterations in diaphragmatic myofibrillar protein concentration, suggest that some step in E-C coupling is critical in explaining the diaphragmatic specific force deficit associated with hypothyroidism.

Although these data do not provide a definitive mechanism(s) to explain which factor(s) related to E-C coupling are altered by hypothyroidism, one potential explanation may be a reduced level of myoplasmic Ca^{2+} available to the myofilaments for activation. Indeed, Simonides and Hardeveld [106] have shown a 30% decrease in sarcoplasmic Ca^{2+} loading capacity from hindlimb muscle homogenates of hypothyroid animals. Given the central role of Ca^{2+} in regulating cross-bridge activation and force generation, a reduction in myoplasmic Ca^{2+} concentration in the vicinity of the contractile apparatus could potentially reduce specific force production in the hypothyroid diaphragm.

In vitro Isotonic Characteristics in the Costal Diaphragm Strip

A second principal finding of the present study is that hypothyroidism reduces maximal shortening velocity and significantly shifts MHC distribution in the costal diaphragm of the adult rodent. This supports our hypothesis that a fast to slow MHC shift and decreased myosin ATPase activity is related to a reduced diaphragmatic V_{max} in hypothyroid animals. The hypothyroid-related decrease in diaphragmatic V_{max} in this study is consistent with preliminary experiments in our laboratory (unpublished observations) and reduced

maximal shortening velocities in locomotor skeletal hypothyroid muscle {5, 12, 33}.

ATP hydrolysis by the myofibrillar ATPase enzyme is thought to be the primary determinant of V_{\max} *in vivo* {89}. Indeed, the maximum rate of crossbridge turnover after the performance of a power stroke determines muscle shortening velocity {7}. Further, in whole skeletal muscle, V_{\max} is influenced by the force-velocity inter-relationship of all fibers comprising that muscle {5}. Thus, in the case of a mixed fiber-type muscle (e.g. costal diaphragm), the rate of ATP hydrolysis (myosin ATPase activity and cross-bridge turnover) is determined by the various myosin ATPase activities (MHC) in all fibers comprising that muscle.

The current study demonstrates an increased type I MHC isoform content in hypothyroid diaphragms, while both type IIb MHC isoform distribution and Ca^{2+} -activated myosin ATPase activity are reduced. In this regard, Hoh {65} has shown that the underlying basis for alterations in Ca^{2+} -activated myosin ATPase activity is an increased or decreased expression of the MHC isoforms. To determine if a fast to slow MHC isoform transition and/or a decreased myosin ATPase activity contribute to the reduced diaphragmatic V_{\max} in hypothyroid animals, we examined the correlational relationship between V_{\max} , MHC composition, and Ca^{2+} -activated myosin ATPase activity. We hypothesized that the underlying mechanism for the hypothyroid-induced decrease in V_{\max} was a reduction in diaphragmatic Ca^{2+} -activated myosin ATPase activity concomitant to a fast to slow MHC isoform shift in the hypothyroid animals. This hypothesis is supported by the significant correlation between V_{\max} and Ca^{2+} -activated myosin ATPase activity ($r=0.80$; $p<0.05$) (figure 4).

While the results of these experiments indicate a significant correlation between V_{\max} and MHC composition (type I; $r^2=0.70$ and IIb; $r^2=0.61$) and myosin ATPase activity ($r^2=0.64$), the coefficients of determination do not explain the entire variation between MHC composition or myosin ATPase activity and V_{\max} . Indeed, the wide range of maximal shortening velocities at a given Ca^{2+} -activated myosin ATPase activity suggest that the reduction in V_{\max} induced by hypothyroidism may, in part, be due to alterations in regulatory contractile protein isoforms (i.e. myosin light chain (MLC), troponin) concomitant with a reduced myosin ATPase activity and fast to slow shift in MHC distribution. For example, Lowey et al. [107] have shown that experimental removal of regulatory and/or alkali light chains reduces *in vitro* acto-myosin shortening speeds without altering myosin ATPase activity. Further, skinned fibers treated to partially remove LC_2 , exhibit decreased maximal shortening velocities without altering isometric tension [108, 109].

Empirical evidence supporting the notion of hypothyroid-induced alterations in regulatory contractile protein isoforms have been reported by several groups. Johnston et al. [51] found a decrease in the proportion of the fast MLC (LC_2f) while Nwoye et al. [49] showed decreases in the percentage of fast light chains, Ca^{2+} -activated myosin ATPase, and actomyosin Mg^{2+} -activated ATPase activity in the hypothyroid soleus. In addition to MLC, hypothyroidism has also been shown to reduce the expression of the fast isoform of troponin I, the subunit responsible for inhibiting actomyosin interaction [110]. Currently, no information is available on the impact of hypothyroid-induced changes in regulatory protein isoforms or sarcoplasmic Ca^{2+} kinetics on diaphragmatic contractile function. Hence, their contribution to the reduced V_{\max} in the present study are unknown.

In vitro Isometric Twitch Characteristics

It is well established that locomotor twitch contractile characteristics are altered in response to hypothyroidism {12, 31, 33, 34, 111}. Our experiments support this notion as diaphragmatic twitch parameters related to both activation (dP/dt ; TPT) and relaxation ($RT_{1/2}$, CT) were altered by hypothyroidism. Given this modulation in diaphragmatic twitch characteristics, what intracellular events are altered to account for these responses?

Tension development occurs as a consequence of Ca^{2+} release from the SR and the binding of Ca^{2+} to troponin {7, 112}. Current thought suggests that dP/dt is limited by the rate of myosin-actin binding; that is the transition rate of the acto-myosin complex from the weakly bound low-force state to the strongly bound high-force state {105}.

Given the hypothyroid-induced decrease in the rate of tension development (dP/dt) observed in the current study, four potential physiological changes could be involved: 1) a slowing or uncoupling in action potential development in fibers of a given motor unit; 2) alterations in action potential magnitude requiring an elevated threshold for the onset of force development; 3) reduced Ca^{2+} sensitivity of the contractile apparatus; or 4) a reduction in the rate of passive Ca^{2+} release from the sarcoplasmic reticulum (SR). Work by Denys and Hoffman {31} indicate no abnormalities in the depolarization or repolarization phase of the action potential in hypothyroid animals; further no delay in nerve conduction was noted. Therefore, alterations in the neural transduction pathway seem unlikely to account for the observed alterations in muscle contractile function. Further, given a normal resting membrane potential and

action potential [31], no direct evidence exists to suggest that hypothyroidism increases the threshold concentration of myoplasmic Ca^{2+} necessary to initiate myofilament shortening. Indeed, our single fiber data would suggest that the ability of the contractile apparatus to generate maximal force is unaltered in response to hypothyroidism (figure 10). Finally, the single fiber force-pCa relationship (e.g. $[\text{Ca}^{2+}]_{50}$ values) (table 5) demonstrates that Ca^{2+} sensitivity of hypothyroid myofibrils is enhanced relative to control fibers, possibly due to an increased distribution of type I fibers. Thus, one may rule out the role of reduced Ca^{2+} sensitivity as a potential explanation for the reduction in dP/dt . Therefore, by elimination we conclude that hypothyroidism may reduce the passive release of Ca^{2+} ions from the SR, possibly by modulating the phospholipid composition of the SR membrane (see below). A change in membrane fluidity could potentially slow Ca^{2+} release from the SR and reduce the rate of activation of the contractile apparatus. Such an alteration may explain the delays in TPT and dP/dt observed in the hypothyroid group. Further, we cannot rule out the possibility that modulation in troponin isoform expression, thereby altering acto-myosin interaction, may also contribute to this finding.

The SR plays an integral role in regulating cytosolic free Ca^{2+} concentration and thus its role in regulating muscle relaxation is well known [113]. Gillis [114] suggests that it is the initial and transitory rate of Ca^{2+} uptake by the SR which accounts for relaxation rate *in vivo*, hence the best overall physiological measure of skeletal muscle relaxation is $\text{RT}_{1/2}$.

Since muscle relaxation is determined by the rate of Ca^{2+} re-uptake by the SR, slowed relaxation of the *in vitro* diaphragm strip suggests a delay in sarcoplasmic Ca^{2+} re-uptake. Evidence supporting thyroid hormone influence on sarcoplasmic reticular Ca^{2+} transport has been provided by Limas [115]

who showed an increase in Ca^{2+} -ATPase activity in isolated myocardial SR vesicles from hyperthyroid rats. In this study, they concluded that increased Ca^{2+} -ATPase activity was responsible for the enhanced rate of Ca^{2+} uptake. Consequently, if Ca^{2+} -ATPase activity is reduced in the hypothyroid state, this could explain the observed prolongation in $\text{RT}_{1/2}$ and contraction time.

Empirical evidence implicating thyroid hormone in the regulation of Ca^{2+} handling in skeletal muscle has been reported by numerous groups. Fitts et al. [112] demonstrated increases in initial rates of sarcoplasmic Ca^{2+} uptake and Ca^{2+} -activated SR ATPase of chronically-treated hyperthyroid soleus muscle. These results were confirmed by Nwoye et al. [49] who showed altered rates of Ca^{2+} uptake by isolated SR vesicles from both hypo- and hyperthyroid soleus muscle. In addition, Simonides and Hardeveld [106] report significant reductions in Ca^{2+} loading capacity, Ca^{2+} uptake activity, and total purified SR yield in the hypothyroid gastrocnemius of the rat. Further, these results indicated a significant decrease in SR content per volume of muscle and an increased energy of activation of the Ca^{2+} transport enzyme, $(\text{Ca}^{2+}\text{-Mg}^{2+})\text{-ATPase}$. Conversely, Pilarska et al. [116] found altered sarcolemmal phospholipid composition and a lower energy of activation for $(\text{Ca}^{2+}\text{-Mg}^{2+})\text{-ATPase}$ in the hindlimb musculature of thyroxine-treated animals.

Alternatively, Fanburg [117], while demonstrating a reduction in sarcoplasmic Ca^{2+} uptake from the hindlimb muscles of thyroidectomized rats, found no relationship between alterations in Ca^{2+} transport and ATPase activity of the SR. Rather, Fanburg [117] postulated that modifications in skeletal muscle SR structure (i.e. protein and/or phospholipid composition) account for the observed hypothyroid-induced reductions in Ca^{2+} transport. Indeed, the ability of thyroid hormone to influence lipid and protein

composition of numerous cellular organelles is well characterized {118-120}. Specifically in skeletal muscle, Simonides and Hardeveld {121} have shown that the SR phospholipid matrix in the fast-twitch portions of the rat hindlimb is modified in response to hypothyroidism. Further, they propose that the reduced activity of the SR (Ca²⁺-Mg²⁺)-ATPase occurs as a result of changes in SR membrane phospholipid composition (e.g. fluidity).

Collectively, the literature suggests that by modifying SR function, through altered membrane composition and reduced Ca²⁺-Mg²⁺-ATPase activity, hypothyroidism alters Ca²⁺ kinetics which modulates twitch and potentially tetanic contractile properties of skeletal muscle.

In vitro Single Fiber Calcium Sensitivity

Myofibrillar Ca²⁺ sensitivity is defined as the Ca²⁺ concentration at which isometric tension is half-maximal and is calculated by the [Ca²⁺]₅₀ of the force/pCa relationship. In control muscle, slow twitch (type I MHC) fibers exhibit a left shift in their force/pCa relationship (greater Ca²⁺ sensitivity) compared to fast twitch (type II MHC) fibers {Fitts et al., 105}. Thus the leftward shift in the force/pCa curve for hypothyroid fibers suggests an increased expression of the slow type I MHC in the myofibril and a transition toward slower contractile characteristics. Indeed, electrophoretic analysis of single fiber MHC content revealed a disproportionate number of fibers expressing the slow, type I MHC in the hypothyroid group. The observation of an increased type I MHC expression in hypothyroid single fibers is consistent with the increased distribution of the type I MHC isoform in diaphragm myofibrillar samples (figure 2). Therefore, it seems likely that hypothyroidism increases the proportion of slow, type I fibers in the costal diaphragm, thus

producing an increased Ca^{2+} sensitivity of those individual type I diaphragm myofibrils. This observation supports previous work in hypothyroid diaphragm [29] and locomotor muscle [5] which demonstrate an increased distribution of slow, type I muscle fibers. Finally, given that variations in single fiber contractile properties (i.e. force-pCa relationship) are correlated with alterations in MLC and troponin isoform expression [122], it is feasible that hypothyroid-induced modulation of regulatory contractile proteins contribute to the increased Ca^{2+} sensitivity of hypothyroid single fibers. However, additional work will be required to verify these postulates.

Summary and Conclusions

Three specific factors are thought to constitute the basis for maximal *in vitro* specific muscle force generation: 1) the concentration of myofibrils in parallel per unit of muscle cross-section, 2) the maximal force generating capacity of an individual cross-bridge, and 3) Ca^{2+} -induced sarcomere activation via the excitation-contraction coupling cascade. These experiments were designed to systematically determine the role of factors #1 and #2 in contributing to the hypothyroid-induced force deficit of the rat diaphragm. These data demonstrate that the observed *in vitro* specific force deficit is not due to a hypothyroid-induced alteration in diaphragmatic myofibrillar protein concentration or reduction in the intrinsic force producing capacity of the contractile apparatus. Therefore, it is concluded that some step in E-C coupling is the principle factor contributing to the hypothyroid-induced diaphragmatic specific force deficit. One possible explanation for the observed *in vitro* force deficit may be a decreased myoplasmic Ca^{2+} concentration

available for myofilament activation. This may be the result of a reduction in Ca^{2+} storage in the SR of the hypothyroid diaphragm strip.

Finally, this study demonstrates that hypothyroidism produces significant shifts in MHC isoform content and Ca^{2+} -activated myofibrillar ATPase activity sufficient to modulate diaphragmatic V_{max} . However, hypothyroid-induced alterations in other regulatory contractile protein isoforms may also contribute to this effect.

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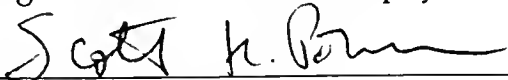
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BIOGRAPHICAL SKETCH

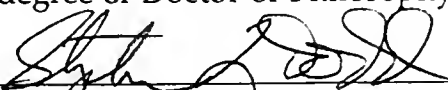
Robert A. Herb was born and raised in the community of Fort Plain, in upstate New York. He graduated from the State University of New York College at Cortland in May of 1985 with a bachelor's degree in physical education and biology. He received his master's degree in health and sport science from Wake Forest University in Winston-Salem, NC, in August of 1987. Upon graduation, he accepted a temporary faculty position in the Department of Health and Physical Education at Humboldt State University in Arcata, CA. In 1989, he began his matriculation at the University of Florida to pursue a Doctor of Philosophy degree in exercise physiology with a minor in physiology. Following graduation, he will accept a position as an Assistant Professor in Exercise Science in the Department of Health, Physical Education, Exercise Science, and Nutrition at Northern Arizona University in Flagstaff, AZ.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



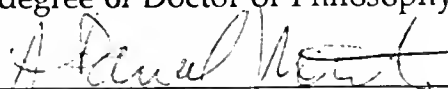
Scott K. Powers, Chair
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Sciences

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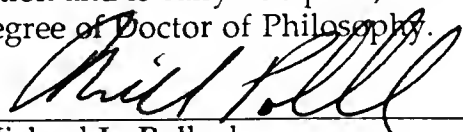
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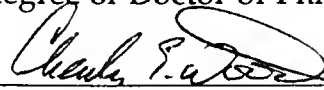
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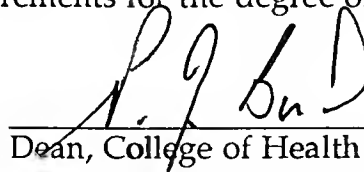
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This dissertation was submitted to the Graduate Faculty of the College of Health and Human Performance and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1994



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